ANALYSIS OF THE INHIBITORY FACTORS AGAINST THE PROTEIN DIGESTIBILITY OF PLANT FOODSTUFFS(Dissertation_全文)

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INTRODUCTION

Legumes and cereals constitute a staple part of dietary protein of large segments of the world's population. On a worldwide scale, approximately 70% of the protein available for human consumption is derived from plant sources (1). Cereal grains contribute about 50% of the total protein consumed; and about 20% of the total proteins comes from oilseeds and legumes (1). Although the production of cereals and legumes could be conceivably expanded to provide a sufficient amount of the total protein needed, nutritionists are well aware of the fact that the proteins in the plant seeds are in general of poor quality. The nutritional quality of the plant seed proteins continues thus to be an important problem in human nutrition. Edible seeds, on the other hand, are barely accepted as fresh vegetables, but are usually subjected to some form of processing or cooking prior to consumption. Although traditional methods of preparation should have served to minimize any adverse physiological effects which accompany by the ingestion of raw seeds, the rationale for such methods of preparation has been not fully elucidated.

While the plant seeds generally contain moderate amounts of protein, they also contain a number of constituents which adversely affect the utilization of the protein present (2). Among the many deleterious factors present in edible seeds, protein protease inhi-

bitors have been most extensively investigated. Studies with laboratory animals have shown that protease inhibitors may inhibit growth, reduce digestibility, and cause pancreatic hypertrophy (2). But, there is not always clear-cut correlation between the protease inhibitor content of various edible seeds and the beneficial effect which cooking and other commom means of preparation has on their nutritive quality.

In this connection, current evidence suggests that there are some other antinutritional factors adversely affecting the biological availability of proteins in many edible seeds. The seeds of many legumes and cereals contain tannic substances. The tannins are known to impair utilization of proteins in human and animal diets by binding with and coagulating protein (3, 4). Growth retardation has been observed in animals fed containing tannins (5). Phytate, a common constituent of plant tissues, has been shown to have an inhibitory action against a proteolytic enzyme (6-8). Lectins, which are present in the seeds of many legumes, may reduce the intestinal absorption of essential nutrients through their combining with the absorptive cell (9).

Evidence has been accumulated indicating that dietary fiber, a major constituent of plant foodstuffs, can express an inhibitory effect on the assimilation of certain essential nutrients from the gastrointestinal tract. Many investigators have observed that

a significant increase in fecal nitrogen excretion occurs on high-fiber diets (10-15). It thus appears that dietary fiber is not innocuous in the alimentary tract, but may reduce the biological availability of dietary protein.

A number of antinutritional factors may reduce the biological availability of the proteins in plant foodstuffs through their inhibitory activities towards the digestion or absorption. However, the correlation between the nutritive values of edible seeds and the overall level of their inherent antinutritional factors is still not clear. The question of what components in plant foodstuffs product most significant inhibition also remains unanswered. Furthermore, there is no consensus of opinion as to the factors responsible for the protein quality of plant foodstuffs.

The investigations presented here were performed for a systematic understanding of the inhibitory factors involved in the protein digestibility of plant foodstuffs. This thesis consists of four chapters: The first chapter aims to identify dietary fiber in plant foodstuffs as an inhibitory factors against protein quality, and the second chapter describes the effects of processing on the protein digestibility and protease inhibitors in edible seeds Possible roles of tannins and phytate as an antinutrient affecting the protein digestibility of edible seeds is discussed in the

third chapter. The final chapter, Chapter IV, aims to clarify the overall inhibitory potency of the plant antinutritional factors towards protein digestibility and to reveal the inhibitory factors responsible for the protein digestibility of plant foods.

CHAPTER I

IDENTIFICATION OF DIETARY FIBER IN PLANT FOODSTUFFS AS AN INHIBITORY FACTOR AGAINST THEIR PROTEIN DIGESTIBILITY

Dietary fiber, which is an abundant component in plant foodstuffs, is all the components of a food that is not broken down by the secretions of the human gastrointestinal tract to produce small molecular compounds which are then absorbed into the blood stream (16). It consists of various proportions of complex carbohydrates such as hemicellulose, pentosans, pectic substances, gums, mucilages, and certain other carbohydrates, as well as the lignin and cellulose (17). There is a distinction between crude fiber and dietary fiber, since the former is a designation of a fraction determined analytically in a manner that gives an approximation of only cellulose and lignin and not of other carbohydrates not digested by man (18).

Over ten years have elapsed since the hypothesis was advanced that dietary fiber is not a physiologically inert substance nor contributes to human metabolism virtually insignificant (19). Now interest in dietary fiber and its physiological effects is growing rapidly. Primary interest has focused on the beneficial effects in the prevention of certain gastrointestinal and vascular diseases (20, 21). Although some of these beneficial effects are not unequivocally accepted, dietary fiber preparation and fiber-enriched foods

are available, and their consumption by the health-conscious public is increasing. In view of this development, information on possibly hazardous effects of dietary fiber is urgently needed. Possible interference with the digestion and absorption of essential nutrients is one area of uncertainty. Several studies with laboratory animals on high-fiber diets have shown a significant increase in fecal nitrogen excretion (10, 14). Increasing the amount of fiber in the diet of humans has also been shown to reduce apparent digestibility (11,12, 15). The activities of digestive enzymes appear to be affected by dietary fiber, perhaps leading to an effect on digestion and absorption in the gastrointestinal tract. Schneeman (22) has indicated that several dietary fiber sources have inhibitory capacities towards some digestive enzymes. Rats fed diets containing high levels of fiber have lower levels of intestinal proteolytic enzymes (23). These studies suggest that dietary fiber is not innocuous in the alimentary tract, but may reduce the availability of dietary protein.

The present chapter describes the identification of dietary fiber as an inhibitory factor against protein digestibility.

MATERIALS AND METHODS

Materials

All the dietary fiber sources examined in this study, except

for hemicellulose from buckwheat flour, were of the highest grade commercially available. Water-soluble hemicellulose from buckwheat flour was prepared by the procedure of Cartaño and Juliano (24) with a slight modification. Proteolytic enzymes used were obtained from Sigma Chemicals Co.: trypsin ([EC 3.4.21.4], 2 X crystalline, from bovine pancreas); α-chymotrypsin ([EC 3.4.21.1], 3 X crystalline, from bovine pancreas); and pepsin ([EC 3.4.23.1], 2 X crystalline, from hog stomach mucosa). The substrates for enzymes were obtained from the following companies: N^{α}-benzoyl-D,L-arginine *p*-nitroanilide (BApNA), from Boehringer Mannheim Co.; benzoyl-L-tyrosine *p*-nitroanilide (BTpNA), from Nakarai Chemicals Co.; Hammarsten's casein, from E. Merck, Darmstadt; and hemoglobin, from Sigma Chemicals Co. All other chemicals used were of analytical grade.

Assay of Inhibitory Activity towards Proteolytic Enzymes

Enzyme reaction were performed in the following buffer solutions: trypsin or α-chymotrypsin, 58 mM Tris-HCl buffer (pH 7.6); and pepsin, in 0.13M HCl-KCl buffer (pH 1.6). The hydrolytic activity of trypsin with BApNA was determined according to the procedure of Erlanger *et al.* (25), and its proteolytic activity with casein and hemoglobin as the substrate according to the procedure of Laskowski (26). The activities of α-chymotrypsin and pepsin were assayed by the procedures described by Rick (27) and Anson (28),

respectively. Trypsin and α-chymotrypsin were dissolved in 10^{-3}M HCl at 48 μg/ml and 100 μg/ml, respectively. Pepsin was dissolved in 0.13 M HCl-KCl buffer (pH 1.6) at 100 μg/ml. Dietary fiber sources examined were dissolved or suspended at 0 - 5 mg/0.5 ml in each buffer solution used in the enzyme reaction. The inhibitory activity of dietary fiber sources against enzymes was determined by the following procedure: 0.5 ml of the fiber-containing solution or suspension was preincubated at 37°C for 10 min with both 0.5 ml of enzyme solution and 1.0 ml of their respective buffers, and the remaining activity was then determined. The control mixture was prepared by replacing the fiber solution or suspension with an appropriate buffer solution.

Determination of Protein

Protein concentration was determined by the procedure of Lowry *et al.* (29). The distribution of protein in column effluents was determined by A_{280} measurements.

Assays of Total Uronic Acid and Degree of Methylation of Pectin

Total uronic acid was measured by the carbazole-sulfuric acid methods (30). The degree of methylation of pectins was determined by the procedure of Hudson and Buescher (31).

Measurement of Viscosity

The viscosity of pectic substances was measured with the Ubbelohde-type capillary viscometer at 30°C, 40°C, 50°C, and 60°C. The pectic substances were dispersed in 0.1 M Tris-HCl buffer (pH 7.5). Graphs of viscosity number (η_{sp}/C, cm^3/g) vs concentration (C, g/cm^3) were made for each pectin at each temperature. The loci of the η_{sp}/C vs C graphs were extrapolated to zero concentration in order to obtain $[\eta]$. The density of the dispersions was calculated from the weight of 10 ml aliquots of each pectic dispersion.

RESULTS

Inhibitory Activities of Dietary Fiber Sources towards Proteolytic Enzymes

All the polysaccharides examined here, except for inulin, exhibited inhibitory activity against trypsin (Fig. I-1). Xylan, apple pectin, agar-agar, and the hemicellulose from buckwheat flour had relatively high inhibitory capacity against the enzyme. Almost of the polysaccharides were found to inhibit the activity of trypsin not only with the synthetic chromogenic substrate but also with protein substrates. In addition, xylan, sodium alginate, pectins, and yeast mannan exhibited a pronounced inhibitory effect on the activity of α-chymotrypsin with casein and with BTpNA as the sub-

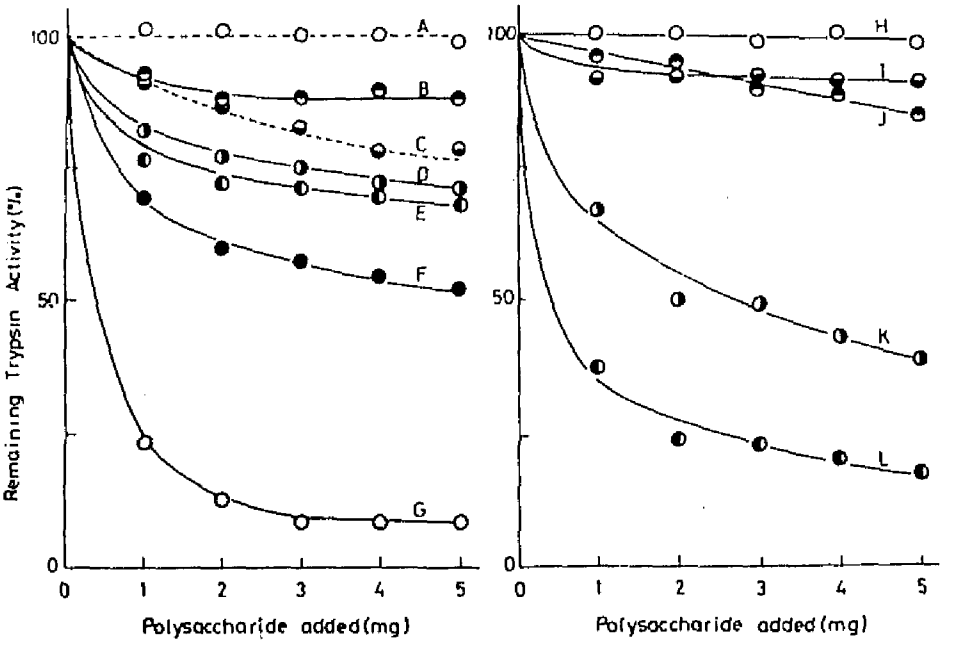

Fig. I-1 Effects of various dietary fiber sources on the activity of trypsin. A, glucuronic acid; B, carboxymethyl-cellulose sodium salt; C, galacturonic acid; D, lemon pectin; E, sodium alginate; F, apple pectin; G, buckwheat water-soluble hemicellulose; H, inulin; I, cellulose powder; J, yeast mannan; K, agar-agar; and L, xylan.

strate; and both pectin (from apple and lemon) exhibited high inhibitory activity towards the activity of pepsin with casein as the substrate (data not shown). On the other hand, galacturonic acid, a major component of pectin, showed an inhibitory activity against trypsin, whereas glucuronic acid exhibited less or substantially no inhibitory activity (Fig. I-1).

The inhibition of trypsin activity by xylan and pectin was estimated with a wide range of the substrate concentration.

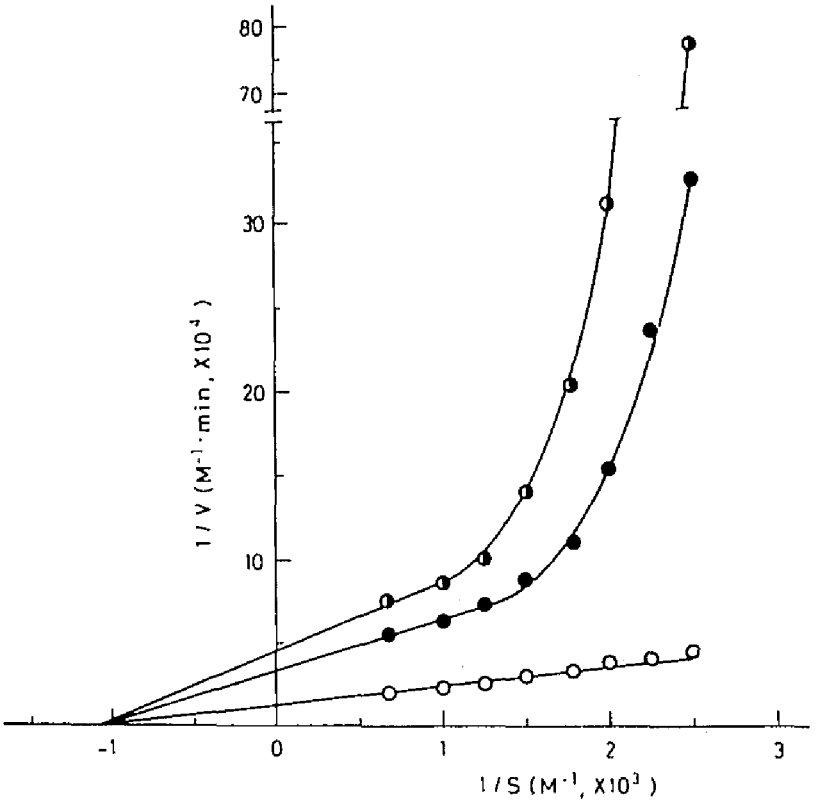

Fig. I-2 Parabolic noncompetitive inhibition of trypsin activity by xylan and pectin.
○——— , without the fiber;
●——— , with pectin (20 mg); and
◑——— , with xylan (10 mg).

Table I-1 Trypsin inhibitory activities and some properties of three kinds of pectic substances from different origins

Pectic substances	Relative trypsin inhibitory activity	Intrinsic viscosity [η]	Huggins coefficient (k')	k'[η]² (X 10^{-3})	Degree of methoxylation (%)
Pectin I	47.6	0.426	5.51 X 10^{-3}	1.0	52.4
Pectin II	28.1	0.329	0.454	49.1	85.0
Pectin III	nil	0.385	0.722	107.0	67.5
Correlation (γ)*	-	0.324_6	-0.969_3	-0.998_7	-0.368_3

* Correlation coefficients for the enzyme inhibitory activity.

Lineweaver-Burk plots showed that these polysaccharides were parabolic noncompetitive inhibitors against the enzyme (Fig. I-2).

Table I-1 shows the trypsin inhibitory activities and some properties of three kinds of pectic substances from different origins. There was no correlation between the enzyme inhibitory activities and the intrinsic viscosity of the pectic substances, as well as their degree of the methylation. On the other hand, the enzyme inhibitory activities of the pectic substances were found to correspond with $k'[\eta]^2$, the activation energy of the viscous flow equivalent (32), and with the Huggins coefficient (33). These findings suggest that the enzyme inhibitory activity of the pectic substances may be closely associated with the distribution of unmethylated carboxyl groups on the polygalacturonic chains.

Interaction of Dietary Fiber Sources with Proteins

Figure I-3 shows the gel filtration chromatographic pattern of hemoglobin incubated with pectin at pH 7.6. Hemoglobin emerged together with pectin, indicating that hemoglobin was able to combine with the polysaccharide under the assay conditions for trypsin and α-chymotrypsin activity. A similar phenomenon was found with sodium alginate.

Figure I-4 shows the interaction of ovalbumin with agar-agar.

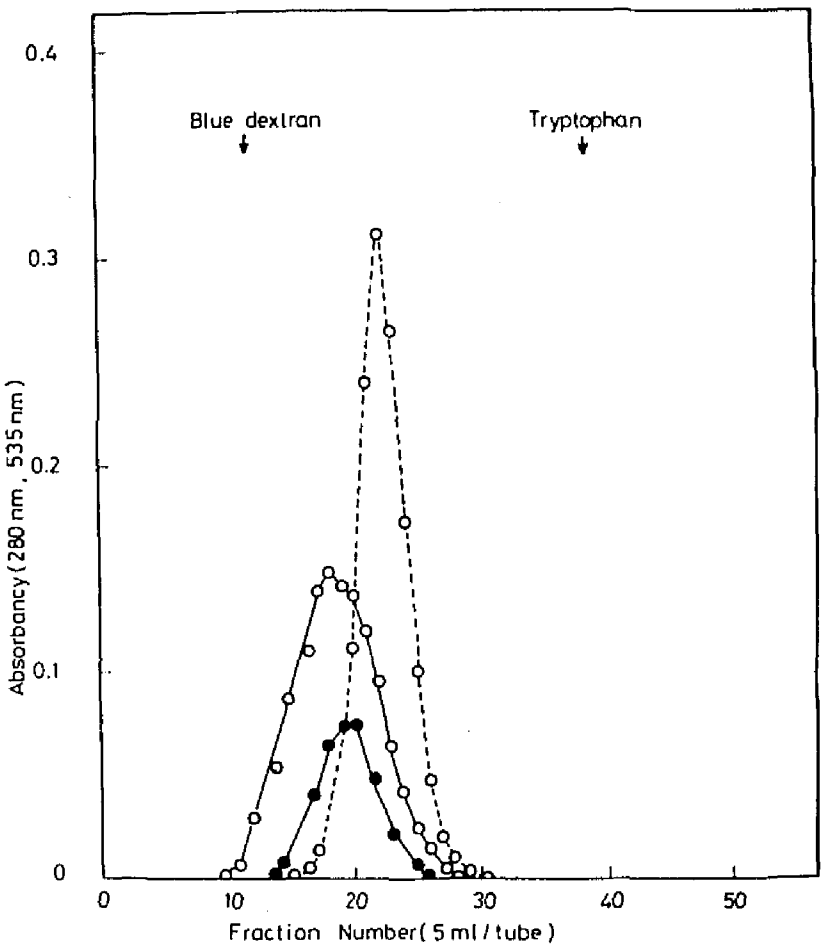

Fig. I-3 Gel filtration patterns of hemoglobin incubated with or without pectin on a Sephadex G-100 column. Five milligrams of hemoglobin was dissolved in 3 ml of 0.1M Tris-HCl buffer (pH 7.6). Pectin solution (10mg/3 ml of the above buffer) was then added to the protein solution. The mixture was incubated at 37°C for 30 min. Aliquots of the incubate were applied on the column (90 X 1.8cm i.d.), which was previously equilibrated with the same buffer.

---○--- , A_{280} in case of hemoglobin alone;

——○—— , A_{280} in case of hemoglobin incubated with pectin; and

——●—— , A_{535} in case of hemoglobin incubated with pectin.

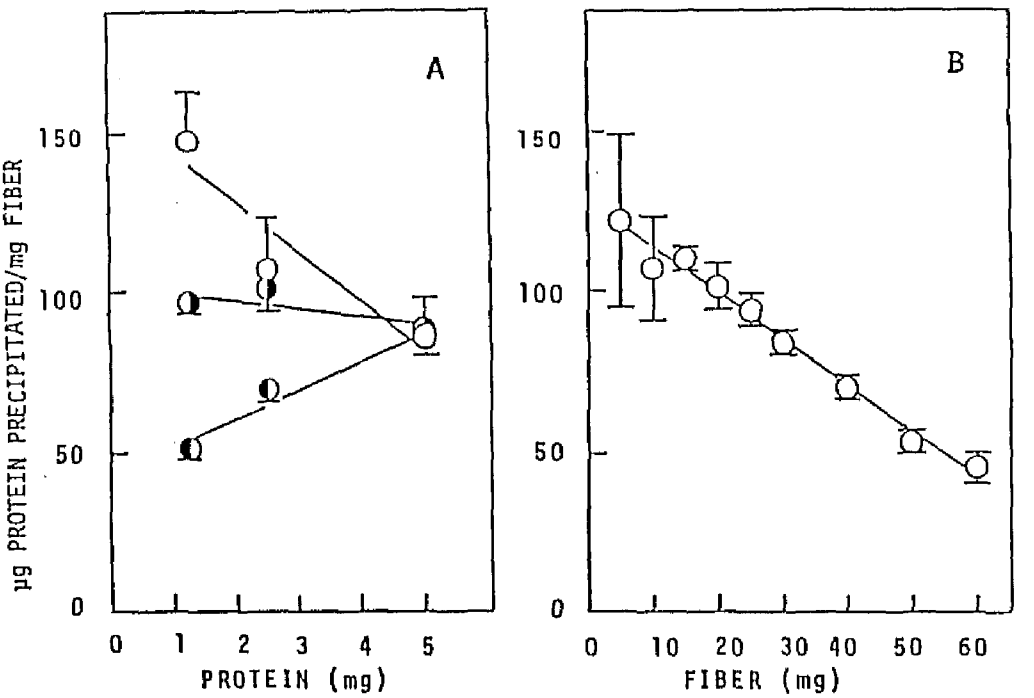

Fig. I-4 Interaction of Ovalbumin with agar-agar.
Ovalbumin (0-5 mg) was incubated with agar-agar (0-60 mg). After incubation, the soluble protein in the incubate was assayed.

——●—— in A, 40 mg of agar-agar;
——◑—— in A, 20 mg of agar-agar; and
——○—— in A, 10 mg of agar-agar.

This polysaccharide exhibited potent precipitating action towards the protein, perhaps through its binding with protein. A similar phenomenon was observed with many of dietary fiber sources such as xylan, carrageenan, agarose, and guar gum. On the other hand, precipitated protein decreased as the concentration of the fiber

source increased (Fig. I-4B). It is noticeable that as the protein concentration increased the differences in the precipitated protein among the concentration of the fiber source was smaller (Fig. I-4A). Hill plot analyses afforded a linear relationship for the binding reaction of ovalbumin with agar-agar with approximately 0.7 of the Hill coefficient; and ovalbumin, which had been previously modified with 2,4,6-trinitrobenzenesulfonate, exhibited virtually no binding capacity towards agar-agar (data not shown). These findings indicate that the binding reaction of the dietary fiber sources with the proteins may occur through the electrostatic interaction on the surface region of both polymer substances.

DISCUSSION

Many dietary fiber sources exhibited significant inhibitory activities towards proteolytic enzymes (Fig. I-2). Kinetic analyses showed that the inhibition of trypsin activity by several dietary fiber sources conformed with a parabolic noncompetitive type (Fig. I-2). Kanaya *et al.* (7) have demonstrated that phytic acid from rice bran, as it combines with the substrate protein, renders the protein poorly available for peptic action, and that phytic acid is a parabolic competitive inhibitor against pepsin. A similar relationship was exhibited between carrageenan and pepsin (34, 35). The observed inhibition of protease activity by the dietary fiber sources may thus

result from the binding with the substrate. The dietary fiber sources substantially combines with protein (Fig. I-3 and I-4). It is, on the other hand, well known that calcium ion stabilizes the activity of trypsin (36). The inhibition of trypsin activity by xylan and pectin became significant as the concentration of calcium ion in the enzymatic reaction mixture was decreased, but became less significant with an increased concentration of this ion (data not shown). Added calcium ion to the assay mixture may combine with the polysaccharides, thus leading to a decrease in the binding capacity of the polysaccharides towards other cationic species, *i.e.*, the substrate. The data obtained in this study indicate that the binding of dietary fiber with proteins may occur through their electrostatic interaction on the surface region of both polymer.

Evidence has currently accumulated indicating that dietary fiber exerts a profound influence upon the digestion of dietary protein. A significant increase in fecal nitrogen was found in laboratory animals on diets containing large amounts of dietary fiber (10, 14). Studies with human subjects demonstrated that high-fiber diets caused a significant decrease in apparent protein digestibility (11,12,15). Several food gums have been shown to substantially lower the *in vitro* digestibility of casein (37). The inhibitory effect of rice bran and alfalfa against trypsin has been reported (22), but the inhibitory mechanism has been not elucidated.

The *in vitro* inhibition of proteolytic enzymes by dietary fiber sources reported here generally agrees with *in vivo* studies (10-15), which show decreases in protein digestibility on high-fiber diets. However, the findings of the present study may not apply directly to a human situation, where there is a continual supply of new digestive enzymes entering the gastrointestinal tract. Within the pancreatic tissue of rats, however, the significant elevation of activities of digestive enzymes, including proteolytic enzymes, was found when wheat bran was added to the diets (38). Increased excretion of endogenous fecal nitrogen has been shown to occur when rats are fed fiber without protein (14). Although the mechanism by which pancreatic enzyme adaptation to diet composition is not fully understood, dietary fiber appears to affect the excretion of digestive enzymes, especially of proteolytic enzymes, through its inhibitory capacity against the enzymes.

CHAPTER II

EFFECTS OF PROCESSING ON THE PROTEIN DIGESTIBILITY AND PROTEASE INHIBITOR IN EDIBLE SEEDS

Among the many biologically active factors present in edible seeds, protein protease inhibitors have most extensively investigated because of the adverse effects they may have on human nutrition. Both the mechanism of action on a molecular level (39) and the nutritional significance of the protease inhibitors (2, 40) have been thoroughly examined.

Inactivation or elimination of the protease inhibitors, without impairing the protein quality of edible seeds, is of particular interest to those concerned with public health and safety. In this connection, it is well known that proper processing and cooking in general lead to an improvement in the protein quality of edible seeds (41, 42). But, the correlation between the improvement of the protein quality and the level of the endogenous protease inhibitor after processing or cooking is still not clear (2).

The present chapter aimed to reveal the effects of processing on the protein digestibility and protease inhibitor in edible seeds. Two different samples consisting of plant seeds as major sources of dietary protein were selected for this investigation: soybean and buckwheat.

SECTION I

Effect of Germination on Protein Digestibility and Protease Inhibitor Level in Soybean

Soybean (*Glycine max* (L.) Merrill) is an important source of dietary protein in some areas of the world, especially in the Far Eastern countries. It is barely eaten uncooked, but is usually subjected to some forms of processing, including soaking and cooking in water, prior to consumption. There are a large variety of soybean foods which are prepared by the Oriental traditional technologies of processing. It is also common practice in many Eastern countries to sprout the soybean for human consumption. In view of rapid growth of interest in the Oriental soybean foods in the Western countries (43-45), the nutritional quality of soybean and its products is a prevalent important subject.

It has been long known that germination improves the nutritive value of soybean, especially its protein quality (46, 47). The total trypsin inhibitory activity appears to decrease during germination (48-50). Conflicting results, however, were reported; any significant change in the antitryptic activity was not observed during germination (51, 52). Thus changes in the protease inhibitory activity during germination are still not clearly understood. Moreover, the correlation between the effect of germination on the protease inhibi-

tor of the seeds and their protein digestibility remains largely uncertain.

The present study was undertaken to examine the changes in the level of protease inhibitors and in *in vitro* protein digestibility during the germination of soybean.

MATERIALS AND METHODS

Materials

Glycine max (L.) var. Tsurunoko soybean, harvested in November, 1984, in Japan, were obtained and stored at 4°C until use. Descriptions on the crystalline trypsin and pepsin preparations used in this study are given in Chapter I. Pancreatin NF was obtained from Difco Laboratories; and α-amylase ([EC 3.2.1.1], from *Bacillus subtilis*), from Sigma Chemicals Co. Commercial crystalline soybean trypsin inhibitor which corresponded with the Kunitz trypsin inhibitor (53, 54) was purchased from Sigma Chemicals Co. The Bowman-Birk trypsin inhibitor was prepared by the procedure described by them (55, 56). Toyopearl HW-50 was obtained from Toyo Soda MFG, Ltd. All other chemicals were of analytical grade.

Germination

Soybean seeds were soaked in deionized water for 5 hr. After soaking, the seeds were sterilized with 0.1% cupric sulfate and then washed with sterilized, deionized water. The seeds were placed in

a humid, dark chamber at 25°C, and allowed to germinate for 9 days. The seeds were sprayed daily with sterilized, deionized water. The seedlings obtained were lyophilized and then ground prior to analysis. The seedling flour obtained was extracted with 10-fold volumes of 0.2 M sodium chloride solution for 2 hr at 4°C and centrifuged at 10,000 X g for 20 min at 4°C. The supernatants obtained were subjected for further analysis.

Assay of Enzymatic and Inhibitory Activities

The assay conditions for the activity of trypsin towards BApNA and for the inhibitory activity against trypsin are given in Chapter I. Trichloroacetic acid (TCA)-soluble trypsin inhibitor activity in soybean seeds and in the seedlings were estimated according to the procedure of Hafez and Mohamed (57, 58). One unit of enzyme activity is defined as the conversion of 1 μmol of substrate per minute. One inhibitory unit (IU) is defined as the number of enzyme unit inhibited under the assay conditions employed.

In Vitro Digestion

In vitro digestion was performed by essentially the same procedure as described by Akeson and Stahmann (59) but with a slight modification. The enzymatic assay consisted of a pH 6.9 hydrolysis for 30 min by α-amylase and of a pH 1.0 hydrolysis for 3 hr

by pepsin, followed by a pH 8.0 hydrolysis for 20 hr by pancreatin. Unless otherwise noted, the enzyme-to-protein ratio, except for α-amylase (500 IU), was 1:10. Sodium azide was added to the pancreatic digestion mixture to a final concentration of 0.025% to prevent growth of microorganisms. After digestion, a 4 ml aliquot of the soluble digesta was added to a test tube containing 1 ml each of 10% sodium tungstate and 0.67 N sulfuric acid (60,61). It was allowed to stand for 10 min, then centrifuged at 4,000 rpm for 15 min. The supernatant obtained was assayed for peptide. Per cent protein hydrolysis was calculated from the ratio of the content of free peptides released upon the digestion to the original content of the protein added to the assay mixture prior to digestion.

Determinations of Protein and Peptide

Protein in solid samples was estimated by the micro-Kjeldahl method (62). The assay conditions for the concentration of protein in aqueous samples and for the distribution of protein in column effluents are given in Chapter I. Peptide content was determined colorimetrically with 2,4,6-trinitrobenzenesulfonic acid (63).

RESULTS

Changes in Trypsin Inhibitory Activity during Germination

Table II-1 shows the changes in the total and TCA-soluble trypsin inhibitory activities during germination of soybean seed and development of the seedling plant. The total trypsin inhibitory activity gradually decreased during germination; and the seedlings after 6th day of germination exhibited significantly ($p<0.05$) decreased trypsin inhibitory activity. In the seedlings, the majority of the inhibitor activity was located in the cotyledon, although approximately 2% of the total antitryptic activity was found in the hypocotyl (data not shown).

Table II-1 Changes in the protease inhibitor activity and soluble protein during the germination of soybean

Germination	Total trypsin inhibitor activity (IU/10 kernels)	TCA-soluble trypsin inhibitor activity (IU/10 kernels)	Soluble protein (mg/10 kernels)
Dormant seeds	207 ± 17^a	3.53 ± 0.85^b	852 ± 58^b
Steeped seeds	210 ± 13^a	3.67 ± 1.32^b	842 ± 67^b
3rd-day seedlings	208 ± 17^a	3.37 ± 0.88^b	943 ± 72^a
6th-day seedlings	189 ± 19^b	3.37 ± 0.26^b	953 ± 68^a
9th-day seedlings	188 ± 25^b	3.83 ± 0.87^a	874 ± 68^b

Values are means $\pm$ S.D. (n=6). Values within a column that do not share a common superscript are significantly different at $p<0.05$.

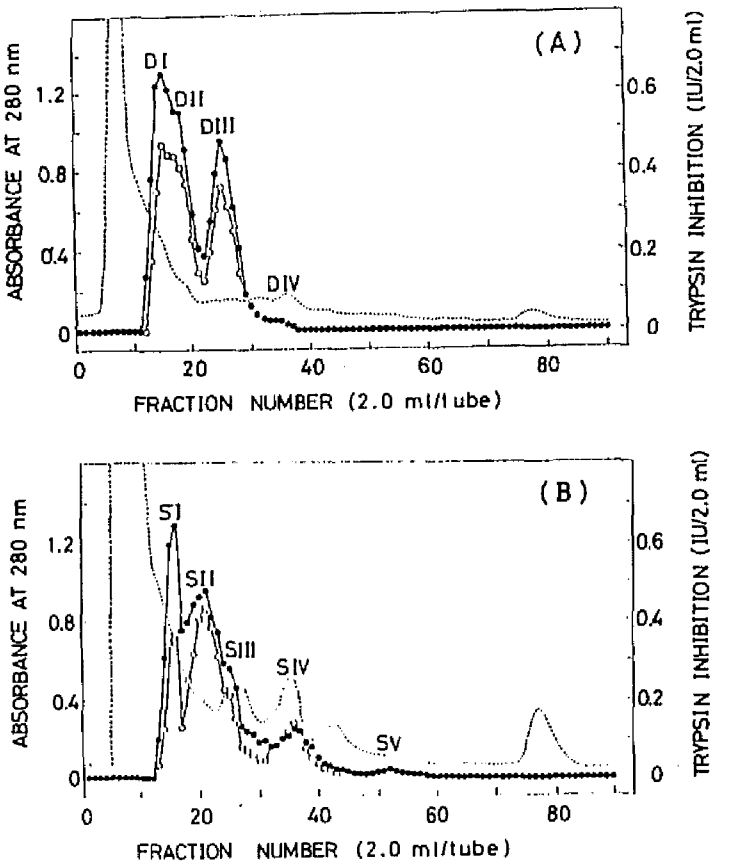

Fig. II-1 Chromatographic elution profiles of the aqueous extracts of soybean dormant seeds and of the seedlings on Toyopearl HW-50. A indicates the aqueous extract of the dormant seeds; and B, the aqueous extract of the 9th-day seedlings. Aliquots of the aqueous extracts were applied on the column (45 X 1.8cm i.d.), which had been previously equilibrated with 0.1 M Tris-HCl buffer (pH 7.2).

—●— , trypsin inhibitor activity;
—○— , trypsin inhibitor activity after heating at 95°C for 1 hr; and
---------- , absorbance at 280 nm.

The TCA-soluble trypsin inhibitory activity comprised approximately 1.7% of the total activity in soybean dormant seed (Table II-1). The TCA-soluble trypsin inhibitory activity declined during germina-

tion, but the reduction rate was rather lower than that of the total activity. Therefore, the concentration of the TCA-soluble trypsin inhibitor of the seedlings for the total activity is higher than that of the dormant seeds.

The elution profiles on gel filtration chromatography on the aqueous extracts from the dormant seeds and from the 9th-day seedlings are presented in Fig. II-1. Four different kinds of inhibitor components were found in the aqueous extract of the dormant seeds (Fig. II-1A), and were designated as inhibitors DI, DII, DIII, and DIV according to their order of elution. The molecular weight was estimated to be 20k, 15k, 8.7k, and 4.8k dalton for the inhibitors DI-DIV, respectively. The inhibitors DI and DIII, as judged from their molecular weight and from the inhibitory activities, may be identical with the Kunitz (53, 54) and Bowman-Birk (55, 56, 64) inhibitors, respectively. This was confimed by subjecting the Kunitz and Bowman-Birk inhibitor preparations to gel filtration under the same conditions employed in Fig. II-1, respectively (data not shown).

Five different kinds of inhibitor components, on the other hand, were found in the aqueous extract of the seedlings (Fig. II-1B), and were designated as inhibitors SI, SII, SIII, SIV, and SV according to their order of elution. The molecular weight was estimated to be 19k, 13k, 9.6k, 4.1k, and 1.2k dalton for the inhibitors SI- SV, respectively. The present findings suggest that the soybean trypsin

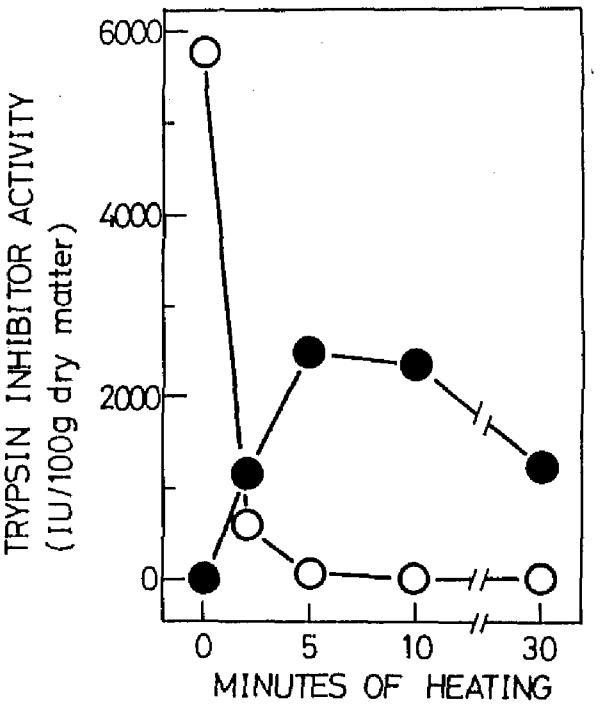

Fig. II-2 Effect of heating on the trypsin inhibitory activity in soybean seedlings. The 9th-day seedlings were assayed. The whole seedlings were subjected to heating in boiling water for various periods, and then the remaining trypsin inhibitory activities in the seedlings and in the soak solutions were assayed.
——O—— , the trypsin inhibitory activity in the whole seedlings;
and ——●—— , the trypsin inhibitory activity in the soak solution.

inhibitor may be modified during seed germination and development of the seedling plant. In addition, it is noticeable that the seedling trypsin inhibitors, especially the inhibitors SII, SIV, and SV, were highly thermostable (Fig. II-1).

Figure II-2 shows the effect of heating on the trypsin inhibitory

activity in the soybean seedlings. The trypsin inhibitory activity present in the seedlings rapidly decreased on immersion heating, and practically disappeared during the first 5-min period of heating. A considerable activity of the trypsin inhibitor was found to be leached out from the seedlings into the soak solution on immersion heating. On the contrary, there was a detectable activity of the trypsin inhibitor in the whole dormant seeds even after thirty minutes of immersion heating under the same conditions as employed in Fig. II-2; and any inhibitory activity was not found in their soak solution (data not shown).

In Vitro Protein Digestibility of Soybean Dormant Seeds and of the Seedlings

Table II-2 shows the *in vitro* protein digestibility of the soybean dormant seeds and of the seedlings. The protein digestibility of the seedlings was significantly ($p<0.01$) higher than

Table II-2 In vitro protein digestibility of soybean dormant seeds and of the seedlings

Soybean sample	Peptic and pancreatic digestibility (%)[1]
Dormant seeds	30.9 ± 1.2^a
Seedlings[2]	53.3 ± 4.6^b

1] Values are means $\pm$ S.D. (n=6). Values within a column that do not share a common superscript are significantly different at $p<0.01$.
2] The 9th-day soybean seedlings were assayed.

Table II-3 Susceptibility of the trypsin inhibitors in soybean dormant seeds and in the seedlings to peptic action

Soybean sample	Remaining trypsin inhibitory activity after peptic digestion[1]	
Digestion period	0.5 hr	1.0 hr
Dormant seeds	62.7 ± 10.6%	61.5 ± 9.2%
Seedlings[2]	64.3 ± 8.5%	58.0 ± 12.4%

1] The aqueous extract of the soybean dormant seeds or of the seedlings was subjected to peptic digestion. Enzyme-to-protein ratio was 1:20. Values are means ± S.D. (n=6).

2] The 9th-day seedlings were assayed.

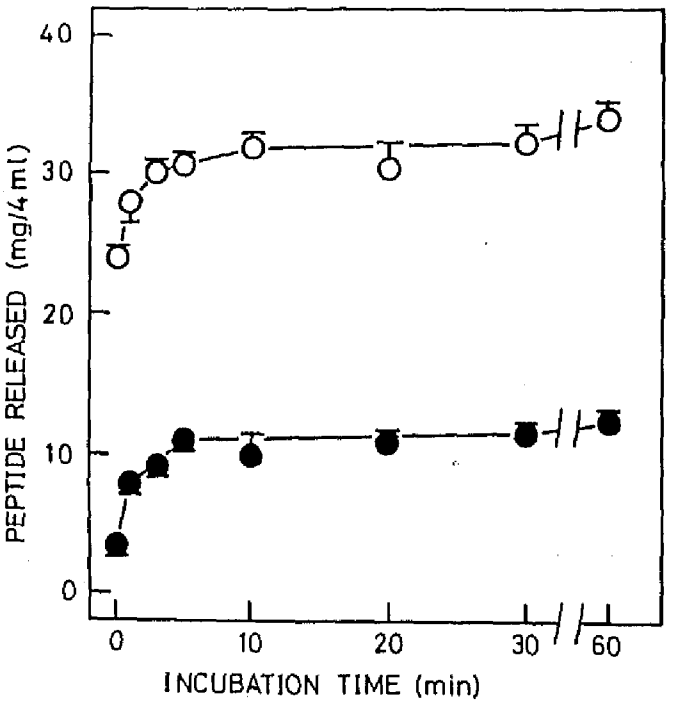

Fig. II-3 Peptic digestion of the proteins in the aqueous extracts from soybean dormant seeds and from the seedlings. The aqueous extracts were subjected to peptic digestion. Enzyme-to-protein ratio was 1:20. The vertical lines in the figure indicate the standard deviation (n=3).
—●—, dormant seeds; and
—○—, seedlings.

that of the dormant seeds (Table II-2), although the seedlings contained a considerable amount of trypsin inhibitor (Table II-1).

Table II-3 exhibits the susceptibility of the trypsin inhibitor proteins in the soybean dormant seeds and in the seedlings to peptic action. There was not any significant difference in the susceptibility of both inhibitor proteins towards peptic action.

Figure II-3 shows the susceptibility of the proteins present in the aqueous extracts of the dormant seeds and of the seedlings to peptic action. Although there is no significant difference between both proteins towards the peptic susceptibility as seen in their initial velocity, a striking difference between the dormant seeds and the seedlings lies in the contents of 2.5% TCA-soluble peptide as shown in the zero time of the peptic hydrolysis (Fig. II-3).

DISCUSSION

The trypsin inhibitor activity gradually decreased during the germination of soybean seed and development of the seedling plant (Table II-1). This finding generally agrees with that of Freed and Ryan (49). Hafez and Mohamed (57,58) have reported the occurrence of a nonprotein (TCA-soluble) trypsin inhibitor, as comprised 21.2 - 55.8% of the total antitryptic activity, in soybean. The present finding indicated that the TCA-soluble trypsin inhibitory activity comprised only approximately 1.7% of the total activity

in the dormant seeds. On the other hand, the concentration of the thermostable, low-molecular-weight inhibitor in the seedlings was virtually higher than that in the dormant seeds (Table II-1 and Fig. II-1). In addition, the soybean trypsin inhibitor may be modified during germination (Fig. II-1). This finding generally agrees with previous observations (49, 65).

It has been long known that the germination of soybean improves the protein quality (46,47). However, the mechanism responsible for the improvement in the protein quality of soybean on germination is still not clarified (2). A level of the antitryptic activity decreased during germination (Table II-1). Furthermore, the majority of the seedling protein, as comprised of approximately 30% of the total protein in the seedlings, consisted of low-molecular-weight components (Fig. II-3). This was also confirmed by polyacrylamide gel electrophoretic analysis of the soybean seedling protein in the presence of sodium dodecylsulfate (data not shown). These findings suggest that the seedling protein in itself may be highly acceptable for the assimilation from the gastrointestinal tract.

In conclusion, the present study suggests that an improvement in the protein quality of soybean during germination may be due to much enhanced acceptability of the seedling protein for the alimentary assimilation rather simply to the decreased antitryptic activity.

SECTION II

Analysis of the Factors Responsible for the Protein Digestibility of Traditional Soybean Foods

In 1982, the revised Standard Tables of Food Composition have been published by the Resources Council, the Science and Technology Agency, Japan (66). In the course of works to estimate the utilizable energy for human of domestic major foods, it has been first shown that there is a distinguishable difference in the protein digestibility for human subjects among various traditional foods prepared from soybean (67). For example, soybean protein-lipid film, yuba, exhibits higher protein digestibility, whereas ground, roasted soybean meals, kinako, considerably lower. Factors responsible for the protein digestibility, as shown in the human experiments, of the traditional soybean foods, however, have been yet not clarified.

The present chapter aimed to reveal the factors involved in the protein digestibility of several soybean foods.

MATERIALS AND METHODS

Materials

Fresh soybean foods were obtained from local markets. Descriptions on the soybean dormant seeds used in this study are given in the Section I of Chapter II. Prior to analysis, the food samples

were lyophilized, then ground finely with an electrically-driven mill, and stored at -35°C until use. Statements regarding enzymes sources and crystalline soybean trypsin inhibitor used in this study are given in the Section I of Chapter II.

Assay of Enzymatic and Inhibitory Activities

The assay conditions for the trypsin activity and for the inhibitory activity against trypsin are given in Chapter I.

In Vitro Digestion

The detailed conditions for the *in vitro* digestion of food samples are given in the Section I of Chapter II.

Determinations of Protein and Peptide

Descriptions with regard to the assay conditions of protein and peptide are given in the Section I of Chapter II.

Determination of Dietary Fiber

Dietary fiber was analyzed by the Van Soest neutral detergent procedure (68) which provided the sum of the contents of cellulose, hemicellulose and lignin in samples examined.

RESULTS

Table II-4 shows the effects of heating and the endogenous trypsin inhibitor on the protein digestibility of soybean seeds. Heating of the soybean seeds, especially autoclaving, leaded to an improvement in the protein digestibility. On the other hand, inclusion of the crystalline Kunitz trypsin inhibitor preparation to the flour of the autoclave-treated seeds to the same level of antitryptic activity as contained in the raw seeds significantly ($p<0.05$) decreased the protein digestibility, but there was a significantly ($p<0.05$) difference in protein digestibility between the fabricated sample and the raw seed. This finding suggests that the protein digestibility of the soybean and its processed

Table II-4 Digestibility of the proteins of raw and heated soybean

Soybean samples	Peptic and pancreatic digestibility (%)[1]	Trypsin inhibitor activity (IU/100g sample)	0.2M NaCl-soluble protein (g/100g sample)
Raw soybean	30.9 ± 1.2^{c}	8502 ± 268	21.3 ± 0.2
Immersion-heated soybean[2]	40.9 ± 3.6^{b}	1080 ± 267	12.1 ± 0.2
Autoclaved soybean[3]	60.6 ± 3.6^{a}	tr.	4.4 ± 0.4
Autoclaved soybean with crystalline trypsin inhibitor[4]	41.4 ± 1.2^{b}	8502	4.4 ± 0.4

1] Values are means $\pm$ S.D. (n=6). Values that do not share a common superscript are significantly different at $p<0.05$.
2] The raw soybean seeds were heated in boiling water for 10 min prior to analysis.
3] The raw soybean seeds were autoclaved at 120°C for 20 min prior to analysis.
4] The flour sample from the autoclaved seeds was mixed with crystalline trypsin inhibitor preparation (6.5 mg per 3g of the flour sample) prior to analysis.

Table II-5 Trypsin inhibitory activities of various soybean foods

Foods	Trypsin inhibitor activity (IU/100g food)	*Protein digestibility*[1]
Raw soybean meal	8502 ± 268	-
KINAKO, Roasted & ground soybean meal	7.4 ± 4.3	*78*
YUBA, Soybean protein-lipid film	249 ± 20	*95*
NATTŌ, Fermented whole soybean	23.2 ± 2.4	*91*
NI-MAME, Boiled whole soybean[2]	61.6 ± 26.5	*91*

1] From the data on the Standard Tables of Food Composition in Japan, 4th rev. ed., by the Resources Council, the Science and Technology Agency, Japan.
2] The whole soybean seeds were cooked under usual recipe *conditions prior to analysis.*

products do not conform to only a function of the trypsin inhibitor activity.

Table II-5 shows the trypsin inhibitory activities of various soybean foods. The processing or cooking of soybean, as shown in this table, reduces the trypsin inhibitory activity. However, there was not any correlation between the endogenous trypsin inhibitor activity in the soybean foods examined here and the protein digestibility which had been estimated on human subjects (67).

Table II-6 shows the digestibility of the protein in various

Table II-6 Protein digestibility of various soybean foods

Soybean food sample	Peptic and pancreatic digestibility[1]
Raw soybean meal	30.9 ± 1.2^{c}
KINAKO, Roasted & ground soybean meal	48.9 ± 3.3^{b}
YUBA, Soybean protein-lipid film	67.3 ± 3.0^{a}
NATTŌ, Fermented whole soybean	64.7 ± 5.2^{a}

1] Values are means ± S.D. (n=6). Values that do not share a common superscript are significantly different at $p<0.05$.

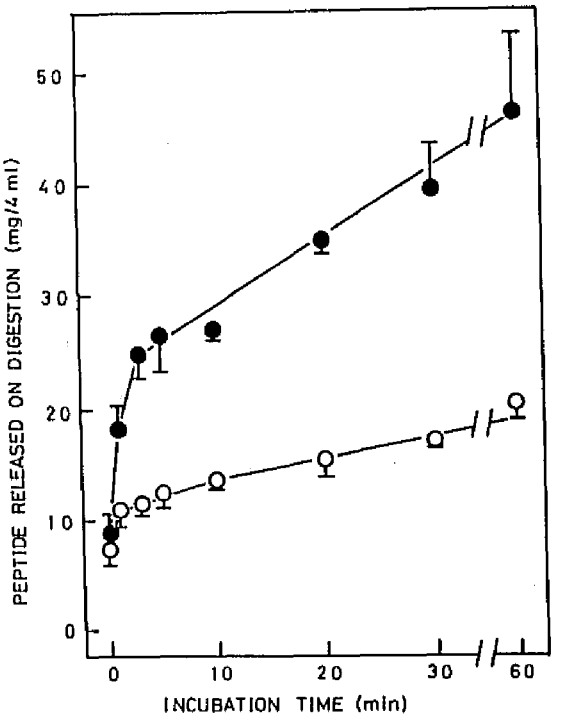

Fig. II-4 Peptic digestion of kinako and yuba. The flour from the samples was subjected to peptic digestion, respectively. Enzyme-to-protein ratio was 1:10. The vertical lines in the figure indicate the standard deviation (n=3).

——○—— , kinako; and

——●—— , yuba.

soybean foods. The *in vitro* protein digestibility of the soybean foods, except for the raw soybean meal, highly agrees with *in vivo* studies on human subjects (67), although such a food as yuba, which had potent inhibitory activity against trypsin (Table II-5), exhibited high protein digestibility (Table II-6).

Figure II-4 shows the peptic digestibility of the proteins in kinako and in yuba. There was a striking difference in the susceptibility of the proteins in the two foods towards peptic action.

DISCUSSION

Protein inhibitors of protease are ubiquitous. They are present in multiforms in many of plant seeds, including soybean, as well as animals and microorganisms (39). Studies with laboratory animals have shown that the protease inhibitors may reduce protein digestibility and produce pancreatic hypertrophy (2). These findings emphasize the physiological and nutritional significance of the protease inhibitors in human health and safety. But, the effect of the protease inhibitors on the nutritive value of foodstuffs is the subject of much controversy.

It was confirmed in this study that the trypsin inhibitor of soybean significantly reduced the protein digestibility (Table II-4). However, it now appears that the protein digestibility of the soybean and its products may not conform to only a function of the trypsin

inhibitor present (Tables II-4 and II-5). On the other hand, the *in vitro* protein digestibility of the soybean foods reported here (Table II-6), highly agrees with the *in vivo* studies on human subjected (67); the correlation coefficient between the *in vitro* and *in vivo* digestibility data was estimated to be 0.995.

Yuba exhibited high protein digestibility; and kinako, less (Table II-6 and Fig. II-4), although there was no relationship between the protein digestibility and the trypsin inhibitor present (Tables II-5 and II-6). On the other hand, kinako contained approximately 12.0% of the neutral detergent fiber, which can express inhibitory potency against proteolytic enzymes (Chapter I), on a dry weight basis; and yuba contained approximately 0.64% of the fiber (data not shown).

The present investigation suggests that, in addition to the protein protease inhibitor, other factor(s) responsible for the protein digestibility of soybean foods, if any, should be taken into consideration. These factors, as well as the protein protease inhibitor, may lead to impaired digestion, absorption, or utilization of dietary protein. This problem will be discussed in the Chapter IV.

SECTION III

Nutritional Characterization of the Components of Buckwheat and Possible Significance of the Endogeous Protease Inhibitor

Buckwheat (*Fagopyrum esculentum* Moench) is an important crop in some areas such as several countries of Europe and of Asia, and USSR of the world. Its potential value as a dietary protein source also is well recognized (69). Most buckwheat seed is usually milled for consumption in pancakes, porridges, or some other flour dishes (70). In Japan, noodles made from buckwheat flour-water dough have long been popular. Buckwheat seedlings or young plants are also available for consumption. Despite the importance of buckwheat as human food, little information is now available on the nutritional properties of the components of buckwheat.

Buckwheat, as is the case with cereals and legumes, is usually subjected to cooking or some other form of heating prior to human consumption. Heat treatment may enhance not only the acceptability for the alimentary assimilation of such a component as starch but also the digestibility of protein. It is, however, uncertain whether or not antinutritional factors, if any, in buckwheat is destroyed during the process of heat treatment.

Although the protein of buckwheat is shown to be of high biological value for animal nutrition (71), its biological availability for

the gastrointestinal assimilation is relatively low in laboratory animals (72-76) and in human subjects (77). The presence of some antinutritional factors in buckwheat seed would seem to affect profoundly the nutritive value.

The present investigation was undertaken to clarify the nutritional properties of the components of buckwheat and to elucidate the factors responsible for the poor protein quality of buckwheat as revealed in animal and human experiments.

MATERIALS AND METHODS

Materials

Fresh and mature buckwheat seeds (*Fagopyrum esculentum* Moench) were obtained locally and stored at 4°C until use. Buckwheat fractions from a commercial mill, SF, 1F, 2F, and 3F, were obtained from a local milling Co.; the fractions are successively prepared by milling buckwheat grain: first fraction is SF (extraction yield 16%); second 1F (40%); third 2F (40%); and the last 3F (3%). Commercial buckwheat straight flour was also obtained locally and stored at -35°C prior to analysis. An artificial tetraploid variety of buckwheat, obtained by the treatment of cochicine, was provided by the author's research group of the Laboratory of Crop Science and Plant Breeding, Shinshu University. Descriptions on the enzyme sources used in this study are given in the Section I of Chapter II. Gel stationary phases

in column chromatography for the separation and analysis of protein were products of Pharmacia Fine Chemicals. All other chemicals used were of analytical grade.

Analysis of Proximate Composition

The assay conditions for protein are given in the Section I of Chapter II. Carbohydrate was determined by the procedure of Bertrand (78). Moisture, ash, and crude fat were analyzed by the corresponding methods of AOAC (62), respectively. Phosphorus was determined according to the procedure of Tanaka *et al.* (79). Other minerals were analyzed with a Hitachi 208 atomic absorbance spectrophotometer after ashing of samples. Thiamine and riboflvin were assayed by the thiochrome method (80) and the lumiflavin method (81), respectively.

Crude fiber was analyzed by the procedure of AOAC (62). Neutral detergent fiber and acid detergent fiber were determined by the procedures of Van Soest (68, 82), respectively. The total content of dietary fiber was determined by the procedure of Asp *et al.*, which can provide the most approximate value to true dietary fiber content among the various procedures (83).

Analyses of Amino Acid and Fatty Acid

Amino acid analysis was performed on a Yanaco LC-8 amino acid analyzer. Protein samples were hydrolyzed at 110°C in 6N HCl for

24 hr in evacuated, sealed tubes (84). Tryptophan was analyzed by the procedure of Spies and Chambers (85) with *p*-dimethylaminobenzaldehyde. Cystine was determined by the performic acid oxidation method (86).

Fatty acid was prepared from samples examined according to the method of Folch *et al.* (87) and then analyzed with a Shimadzu GC-5APF chromatographic apparatus.

Classification of Protein

The total protein of buckwheat flour was classified with respect to different solubility in various solvents (88). Alternatively, the classification of the protein fractions of buckwheat was performed by the procedure of Maes (89), based on their solubility in various solvents, with a column packed with the sample.

Electrophoresis

Disc gel electrophoresis was performed in 7.5% polyacrylamide gel at pH 8.6 by the procedure of Davis (90). Sodium dodecylsulfate polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn (91) in 10% polyacrylamide gel.

Germination

Buckwheat seeds were immersed in 2% sodium hypochlorite solution

and then soaked with deionized water. The seeds were spread thinly on trays, sprayed with deionized water, and kept at 30°C for germination studies. The seedlings obtained were homogenized in a mortar with 10 volumes of 0.2M sodium chloride solution. The homogenates were stirred for 1 hr at 4°C and then centrifuged at 10,000 X g for 15 min at 4°C. The supernatants obtained were subjected to analysis.

In Vitro Digestion

The detailed assay conditions for *in vitro* digestion are given in the Section I of Chapter II.

Assay of Enzymatic and Inhibitory Activities

The assay conditions for the activities of trypsin, α-chymotrypsin, and pepsin and for the inhibitory activity against the proteases are given in Chapter I.

The activity of aminopeptidase was assayed with L-leucine *p*-nitroanilide as the substrate according to the procedure of Apple (92). Enzymes sufficient to give a convenient rate of hydrolysis were added to the reaction mixture which consisted of the following in the final concentration: L-leucine *p*-nitroanilide, 2 mM; and Tris-HCl buffer (pH 7.2), 0.1 mM. The reaction was performed at 35°C for 20 min and stopped by the addition of 1.0 ml of 20% acetic acid solu-

tion. One unit of the enzyme activity is defined as the conversion of 1 μmol of substrate per minute. The hydrolytic activities of the enzyme against other chromogenic substrates coupled with *p*-nitroanilide were determined by measuring of *p*-nitroanilide liberated from the substrates from their spectrophotometer readings at 410 nm; and the activities against L-leucine-β-naphylamide, L-leucinamide, and other peptide substrates were determined by measuring the amounts of L-leucine liberated from the substrates with an amino acid analyzer.

RESULTS

Nutritional Characterization of the Components of Buckwheat

Table II-7 shows the distribution of the nutritive components in buckwheat seed. Many of the nutritive components examined in general were concentrated in the second flour. The first flour contained higher carbohydrate, but less protein and lipid. The minerals and vitamins assayed were also located into the second flour.

Figure II-5 shows the distribution of fatty acids of the lipid fraction in buckwheat seed. Palmitic acid, oleic acid, and linoleic acid were found as the major constituents of the lipid in all the flour fractions of buckwheat. Stearic acid and linolenic acid were also detected in the buckwheat fractions. There were

Table II-7 Distribution of the nutritive components in buckwheat seed

Nutritive component		SF	1F	2F	3F	Whole flour[1]
Protein	(g)	0.69	2.88	15.4	0.98	19.9 (14.6)
Lipid	(g)	0.14	0.76	4.08	0.30	5.3 (1.9)
Carbohydrate	(g)	15.1	36.1	18.0	1.55	70.7 (78.6)
Ash	(g)	0.06	0.36	2.56	0.17	3.1 (1.9)
Calcium	(mg)	1.3	2.8	7.0	0.9	12.0 (17.3)
Phosphorus	(mg)	13.0	86.0	592	35.4	726 (697)
Sodium	(mg)	1.6	4.4	6.4	0.5	12.9 (18.1)
Potassium	(mg)	24.0	100	428	36.9	589 (550)
Magnesium	(mg)	9.6	48.4	440	25.5	524 (550)
Manganese	(mg)	0.1	0.5	2.3	0.2	3.1 (2.7)
Iron	(mg)	0.56	2.8	4.2	0.35	7.9 (7.8)
Copper	(mg)	0.13	0.16	0.56	0.05	0.9 (1.7)
Thiamine	(mg)	0.08	0.32	0.48	0.03	0.91 (0.90)
Riboflavin	(mg)	0.02	0.05	0.10	0.01	0.18 (0.20)
Flour yield	(g)	16	40	40	3	99

1] The whole content of the nutritive component was estimated as the sum of the content of SF, 1F, 2F, and 3F. The values in the parenthesis was obtained by analyzing the whole flour.

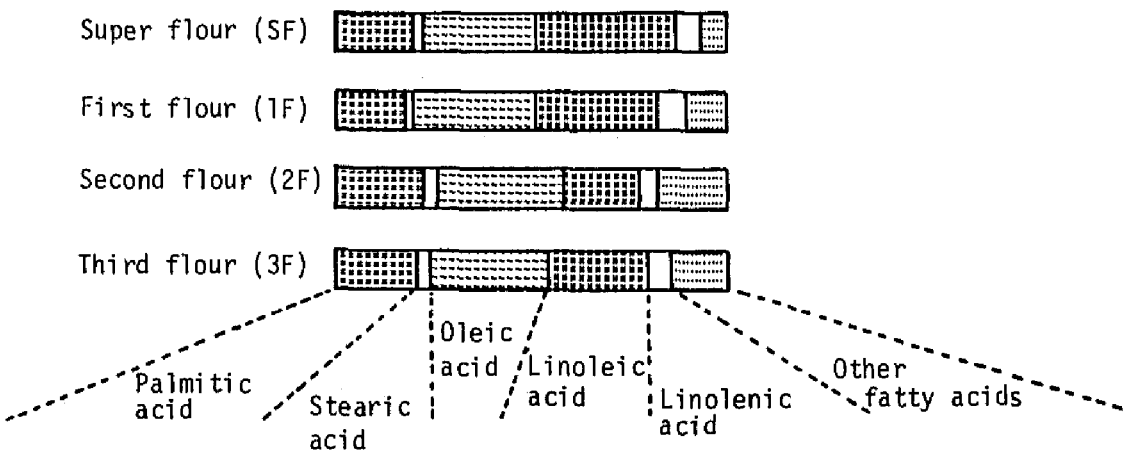

Fig. II-5 Ditribution of fatty acids in buckwheat seed. The indicated areas of the fatty acids were expressed as the per cent of their fatty acids in the total lipid in buckwheat flour.

Table II-8 Dietary fiber of buckwheat flour

Fiber constituent	Fiber content in buckwheat flour (g/100g dry matter)
Crude fiber[1]	2.2 ± 1.2
Neutral detergent fiber[2]	9.1 ± 3.6
Hemicellulose[3]	7.1 ± 3.8
Cellulose[3]	0.9 ± 0.6
lignin[3]	0.9 ± 0.3
Total dietary fiber[4]	14.4 ± 1.3
Soluble fiber[4]	4.3 ± 1.8
Insoluble fiber[4]	10.1 ± 1.5

1] The crude fiber was assayed by the method of AOAC (n=6).
2] The neutral detergent fiber was assayed by the Van Soest detergent method (n=6).
3] Hemicellulose, cellulose, and lignin were estimated from the assayed values of the neutral detergent fiber, acid detergent fiber, and lignin.
4] The total dietary fiber was assayed by the gravimetric, enzymatic procedure (n=3).

fatty acids with 20 or more carbons, fatty acids with 15 or less carbons, and palmitoleic acid as the minor constituents of the lipid of buckwheat.

Table II-8 shows the contents of the crude fiber and dietary fiber of buckwheat flour. The buckwheat flour contained approximately 14.4% of dietary fiber on a dry weight basis and contained approximately 2.2% of crude fiber. A high level of hemicellulose

and a low level of cellulose and lignin characterized the buckwheat flour.

Nutritional Properties of the Proteins in Buckwheat and the Endogenous Protease Inhibitor as an Antinutrient

Figure II-6 shows the classification of the protein components in buckwheat flour with respect to solubility. Representative buckwheat contained 12 to 14% crude protein on a dry weight basis. The

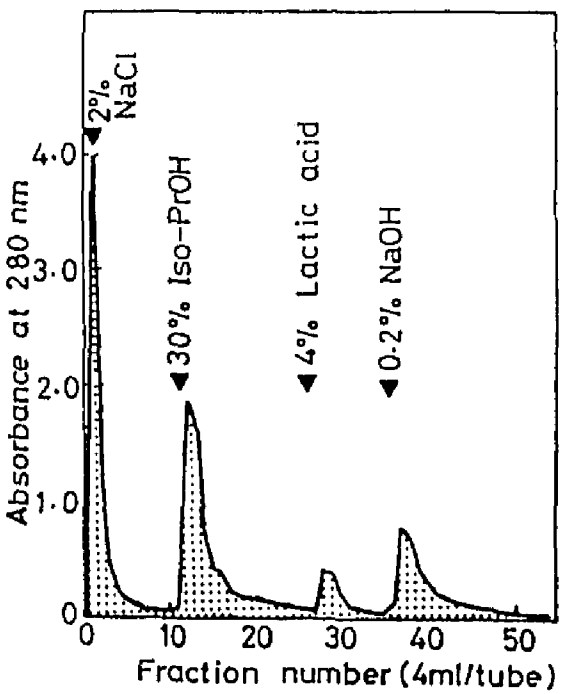

Fig. II-6 Classification of the proteins in buckwheat flour with respect to solubility. The classification was performed according to the procedure of Maes (89). The indicated solvents in the figure were successively poured into a column (9.0 X 1.8 cm i.d.) packed with buckwheat flour between small amounts of sea sand and Celite. The effluents obtained were monitored at 280 nm.

Table II-9 Substrate specificity of the aminopeptidase purified from buckwheat seed

Substrate	Relative activity (%)
L-Leucine *p*-nitroanilide	100
L-Leucine-β-naphtylamide	208.7
L-Leucinamide	19.8
N-Acetyl-L-alanine *p*-nitroanilide	nil
Succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide	nil
S-Benzyl-L-cysteine *p*-nitroanilide	nil
L-γ-Glutamyl-*p*-nitroanilide	nil
α-*N*-Benzoyl-D,L-arginine *p*-nitroanilide	nil
Benzoyl-L-tyrosine *p*-nitroanilide	nil
L-Leucyl-glycine	11.8
L-Leucyl-L-alanine	55.9
Glycyl-L-leucine	17.1
L-Leucyl-glycyl-glycine	8.6

NaCl-soluble protein components (the combined components of albumin and globulin) constituted approximately 60% of the total protein in buckwheat flour (Fig. II-6); and a low level of the components of prolamin and glutelin characterized the buckwheat. A similar finding was obtained by the fractionation of the protein components by the procedure (88) of aqueous, successive extraction from the flour. Each protein fraction had characteristic features in the constituent amino acids: the globulin fraction had a high level of glutamic acid and lysine, and a low level of histidine and serine; the albumin fraction, a high level of glutamic acid and arginine; the prolamin,

a high level of aspartic acid and arginine; and the glutelin, a high level of serine and glutamic acid (data not shown).

The storage proteins of buckwheat seed contribute greatly to its dish product-making quality of buckwheat flour. Any alterations of these proteins such as from proteolytic enzymes would have a profound influence on the quality of resultant products from such flours. In the course of studies devoted to elucidate this problem, preliminary experiments have demonstrated that buckwheat seed should contain a proteolytic enzyme, which is presumably classified as a member of aminopeptidase. The buckwheat aminopeptidase has been purified to a homogeneous state with a recovery of approximately 20%, by the combined procedure of aqueous extraction, affinity chromatography with leucine-bound Sepharose 4B, ion-exchange chromatography with DEAE-Sepharose CL-6B, and chromatofocusing with polybuffer exchanger PBE 94. Table II-9 shows the substrate specificity of the purified aminopeptidase of buckwheat seed. Of the substrates tested, L-leucine-β-naphtylamide demonstrated the highest degree of susceptibility to hydrolysis. In addition, the preferred substrates for the enzyme were L-leucine *p*-nitroanilide, L-leucyl-L-alanine, L-leucinamide, glycyl-L-leucine, and L-leucyl-glycine. The enzyme was also found to liberate L-leucine from L-leucyl-glycyl-glycine but the enzyme did not hydrolyze glycyl-glycine occurring from L-leucyl-glycyl-glycine. No activity towards the other substrates examined were observed (Table

Table II-10 Essential amino acid pattern of buckwheat flour

Amino acid	FAO/WHO pattern (1973)[1] (mg/gN)	FAO/WHO/UNU pattern (1985)[2] (mg/gN)	Amino acid scoring pattern of buckwheat flour	
			FAO/WHO (1973)	FAO/WHO/UNU (1985)
Histidine	-	120	-	123
Isoleucine	250	180	94	131
Leucine	440	410	98	105
Lysine	340	360	109	103
Sulfur-containing amino acids	220	160	97	133
Aromatic amino acids	380	390	111	108
Threonine	250	210	101	120
Tryptophan	60	70	170	146
Valine	310	220	110	155

1] FAO Nutrition Meeting Report Series, No. 52; WHO Technical Report Series, No. 522 (1973) (93)
2] WHO Technical Report Series No. 724 (1985) (94)

II-9). In terms of antagonism by thiol inhibitors such as *p*-chloromercuribenzoate and of non-sensitivity towards chelating agents such as ethylenediaminetetraacetate, the buckwheat aminopeptidase may be a non-metallo, SH-peptidase (data not shown).

Table II-10 shows the essential amino acid pattern of buckwheat. Buckwheat, as shown in this table, consisted of well-balanced amino acids. The protein was particularly rich in lysine. Isoleucine was the first limiting amino acid with ninety-four of the amino acid score. An artificial tetraploid variety of buckwheat had excellent amino acid composition rather than the common diploid variety (data not shown).

The present investigation shows that the protein in buckwheat seed consists of well-balanced amino acid (Table II-10). It has been, however, shown that the biological availability for the gastrointestinal tract of the buckwheat protein is relatively low (72-77). In the course of studies devoted to elucidate the inhibitory factors responsible for the poor bioavailability as revealed in animal and human experiments, the present author came to recognize that buckwheat seed

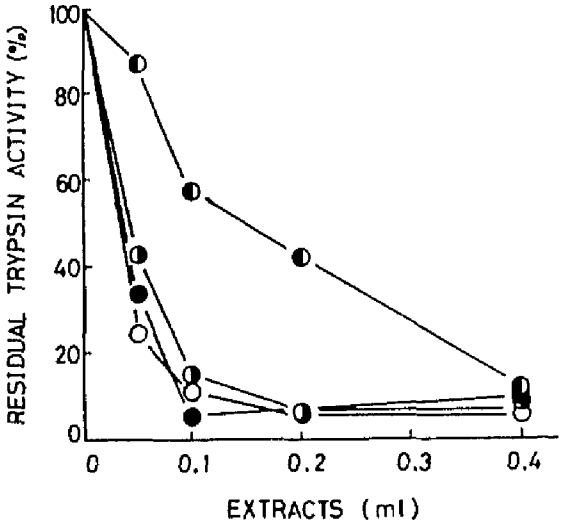

Fig. II-7 Effect of the aqueous extracts from commercial buckwheat flour fractions on the activity of trypsin. The flour fractions were extracted with 0.02 M sodium acetate buffer (pH 4.5) for 2 hr at 4°C with a buffer-to-flour ratio of 10:1. The aqueous extracts obtained were heated for 10 min at 70°C to inactivate any proteolytic enzymes present and were subsequently assayed for trypsin inhibitory activity.

—●— , SF fraction;
—◑— , 1F fraction;
—●— , 2F fraction; and
—○— , 3F fraction.

should contain a protease inhibitor as an antinutrient. Attempts to clarify the occurrence of a protease inhibitor in buckwheat were first performed using the aqueous extract.

Fig. II-7 shows the effect of the aqueous extracts from commercial buckwheat flour fractions on the activity of trypsin. Increased inclusion of the aqueous extracts of the buckwheat flours progressively decreased the hydrolytic activity of trypsin with BApNA as the substrate. The heat treatment of the aqueous extracts prior to the assay of the enzyme inhibitory activity, described in the legend of Fig. II-7, was necessary since the aqueous extracts of buckwheat flour exhibited potent BApNA-hydrolytic activity. Heat treatment of the aqueous extracts for such a short period affected little their inhibitory capacity against trypsin; even after prolonged heating for 2 hr at 98°C, the aqueous extracts retained approximately 91% of the original activity. On the other hand, assays of the inhibitory activity against trypsin in the milling fractions indicated that the enzyme inhibitory activity distributed uniformly in the whole buckwheat seed, although relative low activity of trypsin inhibitor was found in the SF fraction (Fig. II-7).

For the preparation of the trypsin inhibitor, the aqueous extract from buckwheat whole seed, which had been heated for 10 min at 70°C, was salted out with ammonium sulfate to give 80% saturation. A majority of the trypsin inhibitor activity was recovered as a precipitate at this step. The precipitate was collected and then redissolved in

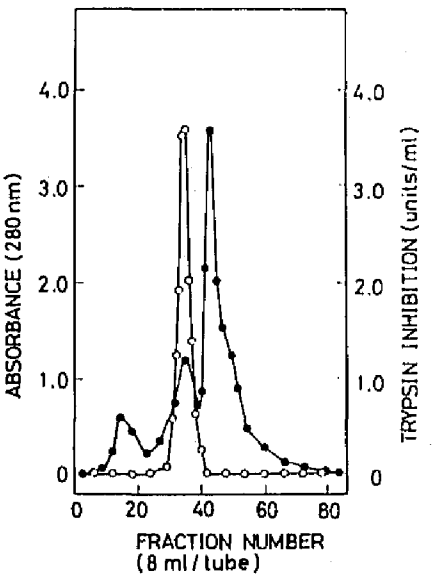

Fig. II-8 Gel filtration chromatography of the active fraction, obtained by fractionation of the aqueous extract of buckwheat flour with ammonium sulfate, on a Sephadex G-75 column (96 X 3.2 cm i.d.). The detailed conditions for the preparation of the active fraction are given in the text.
——○——, trypsin inhibitory activity; and
——●——, absorbance at 280 nm.

Table II-11 Inhibitory activities of the protease inhibitor of buckwheat seed towards various proteases

Proteases tested	Inhibition degree[1]	Substrates used
Trypsin	0.9 μg	BApNA
Trypsin	0.7	casein
α-Chymotrypsin	3.4	casein
Pepsin	>140	casein
Papain	>140	casein
Ficin	>140	casein
Nagarse	>140	casein

1] The amount of the inhibitor required for half inhibition on the activity of 1 μg enzyme. The inhibitor preparation, obtained in Fig. II-8, was assayed.

a minimum volume of the extractant buffer solution. Aliquots of the solution obtained was then applied to a Sephadex G-75 column (Fig. II-8). The inhibitory activity against trypsin emerged as one peak fraction. This active fraction was collected and then assayed for further analysis.

Table II-11 shows the ability of the buckwheat protease inhibitor to inhibit various proteases. The inhibitor strongly inhibited the activity of trypsin with both BApNA and casein as the substrate. The inhibitor also exhibited high inhibitory capacity against α-chymotrypsin, but little or practically no inhibitory capacities against pepsin, papain, ficin, and Nagarse. Another experiments, on the other hand, have been performed to clarify the susceptibility of the buckwheat trypsin inhibitor to peptic action under acidic conditions. It was shown that pepsin was able to digest to the extent of destroying approximately half of the inhibitory activity against trypsin (data not shown).

Attempts were then performed to purify the trypsin inhibitor from buckwheat seed: the inhibitor was isolated from the aqueous extract of buckwheat flour by affinity chromatography on trypsin-Sepharose 4B; and three major inhibitor components were then obtained in a homogeneous state, as judged on polyacrylamide gel electrophoresis, by subsequent chromatography on DEAE-Sepharose CL-6B (data not shown). The molecular weight of the three inhibitor components,

Table II-12 Trypsin inhibitory activity in commercial buckwheat foods

Buckwheat food	Trypsin inhibitory activity (IU/100g dry matter)[1]
SOBA-KO, Raw buckwheat flour[2]	803 ± 50
SOBA-KIRI (KAN-MEN), Raw buckwheat noodles[3]	727 ± 71
SOBA-KIRI (YUDE-MEN) Boiled buckwheat noodles[4]	504 ± 104

1] Values are means ± S.D. (n=3).
2] A commercial buckwheat straight flour was assayed.
3] Commercial buckwheat noodles, consisted of only buckwheat flour (10-WARI KAN-MEN), were assayed.
4] The above raw buckwheat noodles were cooked under usual recipe conditions, then lyophilized, and assayed.

as determined by gel filtration chromatography on Sepharyl S-200, ranged from 8,500 dalton to 9,100 dalton. The trypsin inhibitors obtained had relatively similar amino acid composition; a high level of acidic and basic amino acid residues and a low level of methionine, tyrosine and phenylalanine characterized the inhibitors (data not shown).

Table II-12 shows the effect of cooking on the trypsin inhibitory activity in buckwheat foods. The trypsin inhibitor was highly thermostable: a significant activity of trypsin inhibition remained even after cooking under usual recipe conditions.

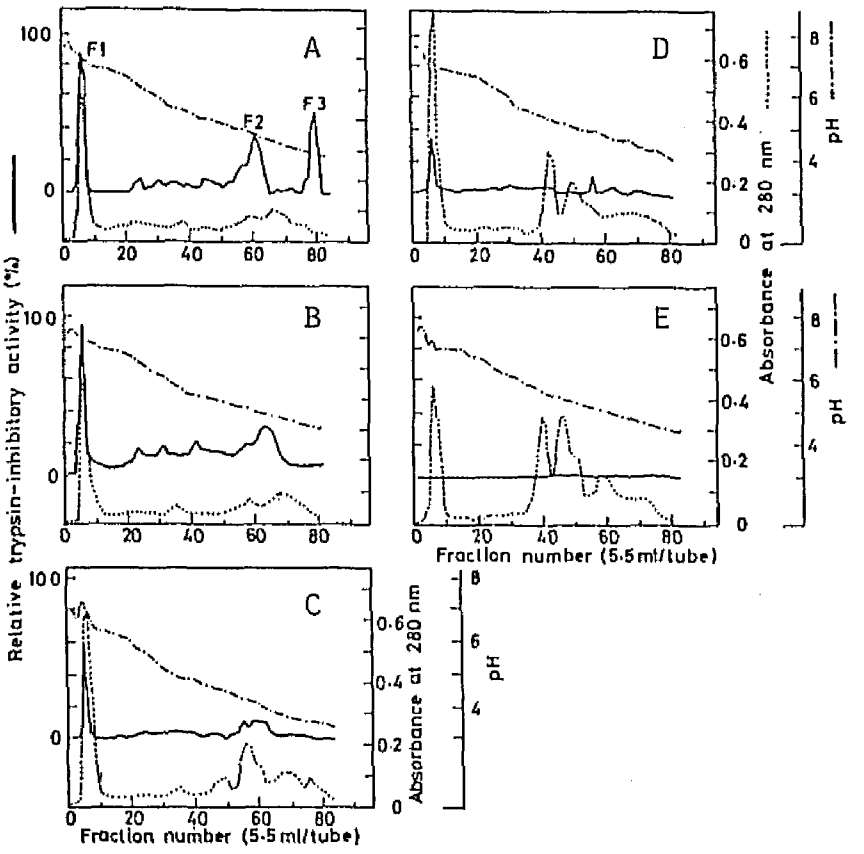

Fig. II-9 Chromatofocusing of the aqueous extracts from buckwheat dormant seed and its seedling on polybuffer exchanger PBE 94. The flour of the dormant seed or of the seedlings was extracted with 10 volumes of 0.2 M sodium chloride solution for 1 hr at 4°C. The aqueous extracts obtained were applied on a column of polybuffer exchanger PBE 94 (35 X 1.0 cm i.d.), pre-equilibrated against 25 mM Tris-HCl buffer (pH 7.2), followed by development with polybuffer 74 at pH 4.0. A, dormant seed; B, seedling on the first day after germination; C, seedling on the second day; D, seedling on the third day; and E, seedling on the fourth day.

Figure II-9 shows the changes in the trypsin inhibitory activity in buckwheat seeds during germination. The trypsin inhibitory activity rapidly decreased after germination. On the fourth day of germination, the seedling had little or no detectable amount of the trypsin inhibitor.

DISCUSSION

Buckwheat is an important source for supplying many essential nutrients, including protein, for human. Buckwheat is the fruit of a dicotyledonous plant and taxonomically distant from the true cereal (69). The plant seed, however, have a number of chemical characteristics in common with cereal grains, especially in relation to its protein concentration, starchy endosperm, and oily embryo (95). In practical commerce, buckwheat has traditionally been classified with the cereals (69).

Many of the nutritive components, including protein, lipid, and several kinds of minerals and of vitamins, were localized in the second flour, the major, outer part of buckwheat grain (Table II-7). The first flour (the major, inner part of the grain), which a popular product of buckwheat in Japan, the so-called Sarashina-noodles mainly consists of, contained higher carbohydrate but less protein, lipid, and some other nutrients (Table II-7). Buckwheat flour contained approximately 1.9% of crude lipid; and palmitic acid, oleic acid, and linoleic acid were found as the major constituents of buckwheat lipid

It has been long believed that a certain constituent, presumably corresponds to the dietary fiber, in buckwheat may have a beneficial effect to progress smoothly the intermediate metabolism of dietary constituents in the intestinal lumen. Characterization of the dietary fiber in buckwheat will afford information on such a beneficial effect for human health. Chemical analyses revealed that buckwheat flour contained approximately 14.4% of dietary fiber on a dry weight basis (Table II-8). The classical crude fiber expressed only about 15% of the total dietary fiber in buckwheat. In addition, a high level of hemicellulose and a low level of cellulose and lignin characterized the buckwheat flour (Table II-8).

Representative buckwheat flour contained 12 to 14% crude protein on a dry weight basis. The protein of buckwheat flour, as is not with cereals such as rice, wheat, and barley (96), consisted of approximately 60% of the salt-soluble proteins. The salt-soluble proteins consisted of approximately 39% globulin, of approximately 30% albumin, and of the other which was dialyzable. On the other hand, buckwheat seed is usually stored for varying lengths of time before consumption. Prolonged storage of the seed might exert a profound influence upon the quality of its resultant products. Studies on the model storage of buckwheat seed have shown that a significant increase in the salt-soluble protein is found, particularly under high temperature and high humidity, after 5.5 months of

storage (data not shown).

An aminopeptidase was purified from buckwheat seed by the combining chromatographic procedure (Table II-9). The enzyme, with L-leucine *p*-nitroanilide as the substrate, exhibited a pH optimum of 7.2; and the *Km* value was 140 μM (data not shown). The preferred substrate were L-leucine-β-naphtylamide and L-leucine *p*-nitroanilide (Table II-9). Studies with enzyme inhibitors suggest that the buckwheat aminopeptidase may be a non-metallo, SH-peptidase. The aminopeptidase, on the other hand, exhibited hydrolytic activity against the endogenous salt-soluble protein of buckwheat (data not shown). This suggests that the enzyme can be a factor affecting the dish product-making quality of buckwheat flour.

The present investigation shows that the protein in buckwheat seed consists of well-balanced amino acids (Table II-10). There are some reports indicating the high quality of the protein of buckwheat (97-99). The nutritive value of dietary proteins in general depends on the biological availability of their amino acids. The presence of antinutrients, as well as the relative proportions of the constituent amino acids, would profoundly affect the protein quality of edible seeds.

In this connection, a number of protein protease inhibitors have been isolated from the seeds of various plants, particularly of legumes (2). Although Laporte and Trémolières (100) have reported

that the flours of some cereals, including buckwheat, and their aqueous extracts exhibit inhibitory activities against trypsin and chymotrypsin, the identity to a protease inhibitor in buckwheat seed remains uncertain. In the present section, a proteinaceous protease inhibitor was demonstrated in the seed of buckwheat (*Fagopyrum esculentum* Moench). The seed of tartary buckwheat (*Fagopyrum tatarium* Gaertner) also had potent inhibitory capacity against trypsin (data not shown). The protease inhibitor was isolated from the aqueous extract of the commom buckwheat seed (*F. esculentum* Moench). The major inhibitor components were purified to a homogeneous state from the seed by the combined chromatographic procedure. The inhibitor protein comprised approximately 0.1% of the whole protein in the buckwheat seed. In addition, the activity of trypsin inhibitor distributed uniformly in the whole seed (Fig. II-7), although relative low activity of trypsin inhibitor was found in the SF fraction, the most inner part of the seed.

The trypsin inhibitor of buckwheat was highly thermostable ; even after cooking under usual recipe conditions, approximately 63% of its original activity remained (Table II-12). This finding was in a striking contrast with as shown in soybean (Table II-5 of the Section II of this chapter). On the other hand, the activity of trypsin inhibitor in buckwheat decreased substantially as germination proceeded (Fig. II-9). On the fourth day of germination, the

seedling had no detectable amounts of the trypsin inhibitor. The susceptibility to peptic and pancreatic hydrolysis of the protein of the fourth day seedling concomitantly was significantly greater than that of the dormant seed (data not shown).

Although buckwheat seed is a source of well-balanced protein (Table II-10), the biological availability of the protein in buckwheat in animals is relatively low (72-76). Studies with human subjects have shown that the digestibility of the protein in buckwheat flour is considerably lower than those in other cereals such as wheat (77). A possible candidate for the factors responsible for the poor protein availability of the buckwheat may be the protein protease inhibitor. However, in addition to the protein inhibitor, other inhibitory factors should be taken into account (the Section II of this chapter). The identity to their overall inhibitory factors will be discussed in the following chapters.

CHAPTER III

POSSIBLE ROLES OF TANNINS AND PHYTATE AS AN ANTINUTRIENT AFFECTING THE PROTEIN DIGESTIBILITY OF EDIBLE SEEDS

It now appears that an enhancement in the protein digestibility of edible seeds on processing or cooking cannot be attributed solely to the removal of the protein inhibitors. Apart from the protein protease inhibitors, there are other deleterious substances, which appear to be universally distributed among the plant kingdom, against protein digestibility. In this connection, current evidence suggests that the broad range of chemical functional groups that compose the amino acid side chains on protein molecule may be responsible for the tendency of proteins to interact with other constituents found in the seeds (101). Such an association is more likely when the seeds are processed for preparation of flours and protein isolate, so perhaps leading to changes in protein quality.

One group of plant constituents capable of interacting with protein may be tannins. Tannins are plant secondary substances that are characteristically rich in phenolic hydroxyl groups. The tannins appear to exhibit a wide variety of biological effects thought to be caused by their capacity to bind with and coagulate proteins (102). Growth retardation has been observed in animals fed diets containing tannins (5). Efforts invested in clarifying the biological effects

of tannins are undoubtedly justified (102, 103), but there is not a clear understanding of the adverse influence of tannins upon the digestibility of proteins.

Phytic acid (phytate), *myo*-inositol 1,2,3,4,5,6-hexakis, is one of the widespread occurrence in plant foodstuffs, particularly in plant seeds. Phytic acid has been shown to form complexes with proteins (104-107), although nutritional implications of phytate-protein complexes are still under scrutiny (108). Some studies showed that phytic acid exhibited inhibitory potency towards the activity of pepsin (6-8).

The present chapter was undertaken to reveal the inhibitory potency of tannins and phytic acid against protein digestibility, and was discussed on possible roles of these substances as an antinutrient.

MATERIALS AND METHODS

Materials

Tannic acid was obtained from E. Merck, Darmstdt; and catechin, from Nakarai Chemicals, Ltd. Phytic acid sodium salt (isolated from corn) was obtained from Sigma Chemicals Co. Descriptions on buckwheat seed used in this study are given in the Section III of Chapter III. Soybean protein isolate (Fuji PRO-R) was kindly provided from Fuji Seiyu Co., Ltd. Crystalline β-lactoglobulin was obtained from Miles Laboratories, Inc.; and gliadin (isolated from wheat), from

ICN Pharmaceutical, Inc. Descriptions on other substrate proteins employed for protease assay and on the proteases used in this study are given in Chapter I. Sephadex G-50 was a product of Pharmacia Fine Chemicals. All other chemicals used were of analytical grade.

Assay of Enzymatic and Inhibitory Activities

The assay conditions for enzymatic and inhibitory activities are given in Chapter I.

Determination of Protein

The assay conditions for protein are given in Chapter I.

Determination of Tannin

Tannin content was determined colorimetrically by the vanillin-hydrochloric acid method (109, 110). The amount of tannin measured was expressed as catechin equivalent.

RESULTS

Inhibitory Potency of Tannins against Trypsin

Tannic acid exerted a relatively high inhibitory effect on the activity of trypsin: kinetic analyses showed that tannic acid was a noncompetitive inhibitor against trypsin (Fig. III-1). The inhibitor contant, K_i, of tannic acid against the activity of trypsin was

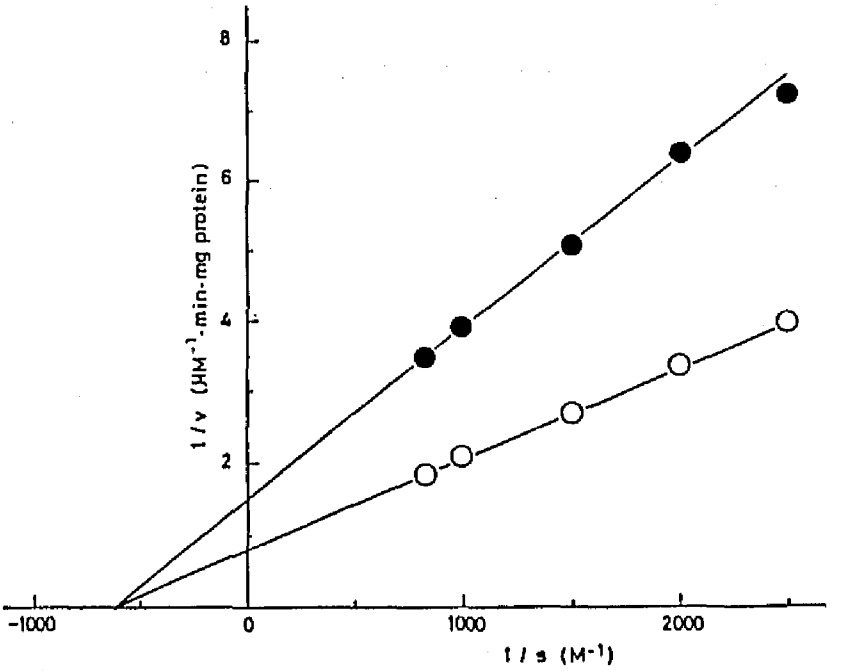

Fig. III-1 Noncompetitive inhibition of trypsin activity by tannic acid. The control reaction mixture contained 1.0 nmol of trypsin, the indicated concentration of BApNA, 980 μmol of Tris-HCl buffer (pH 8.2), and 150 μmol of $CaCl_2$ in a total volume of 4.0 ml.
—○— , without tannic acid; and
—●— , with tannic acid (900 μg).

estimated to be 190 μM. On the other hand, catechin, as assayed with BApNA as the enzyme substrate, exhibited less or substantially no inhibitory activity against trypsin.

Table III-1 illustrates the tannic inhibitory activity against trypsin in buckwheat flour. The tannic trypsin inhibitory activity constituted approximately 16 - 22% of the total trypsin inhibitory activity in buckwheat flour. The molecular weight of this tannic

Table III-1 The total and tannic inhibitory activity against trypsin in various buckwheat seeds

Buckwheat sample[1]	Total trypsin inhibitor (TI) activity (IU/100g flour)	Tannic trypsin inhibitor (TI) activity (IU/100g flour)	Per cent of tannic TI activity in total TI activity
Commercial straight flour	803 ± 50[2]	126 ± 37[2]	15.7 ± 4.4[2]
Seed I	780 ± 10	173 ± 20	22.3 ± 2.6
Seed II	723 ± 3	126 ± 23	17.7 ± 3.6
Seed III	776 ± 40	173 ± 30	22.3 ± 2.2
Seed IV	790 ± 17	173 ± 29	21.7 ± 3.8

1] Fresh commercial straight flour was assayed for the enzyme inhibitory activity; the buckwheat seeds were dehulled, then ground, and assayed. All the seeds used were harvested in Autumn, 1983. The seed I was *F. esculentum* M. var. Shinano-Ichigo; and the other seeds were not identified. The seed I was harvested in Nagano-prefecture, Japan; the seed II, in Hokkaido-prf.; the seed III, in Hyogo-prf.; and the seed IV, in Quebec, Canada.

2] The values presented are means ± S.D. (n=3).

trypsin inhibitor, as determined by gel filtration chromatography on Sephadex G-50, was estimated to be in a range from 800 dalton to 2,000 dalton. The inhibitor was highly thermostable and was positive to vanillin-hydrochloric acid test (data not shown). On the other hand, the tannin content of dehulled buckwheat flour, as assayed by the colorimetrical procedure with vanillin, was estimated to be approximately 1.3 g catechin equivalent per 100 g of flour on a dry weight basis.

Table III-2 Inhibition of the activities of proteases by phyates

Substrate protein / Enzyme tested	Remaining enzyme activity (%)[1]	
	Pepsin	Trypsin
Hemoglobin	98.3 ± 4.1	93.4 ± 8.7
Soybean protein isolate	94.4 ± 4.1	77.8 ± 0.7
β-Lactoglobulin	101.1 ± 4.0	57.3 ± 1.2
Casein	86.6 ± 1.8	99.1 ± 2.5
Gliadin	96.4 ± 6.3	71.4 ± 6.1

1] Values are means ± S.D. (n=3). The enzyme was incubated with phytate at 37°C for 10 min, followed by the addition of the solutions of the indicated substrate proteins. The remaining enzyme activity was determined at 37°C for 30 min. The enzyme-to-substrate ratio was 1:5; and the enzyme-to-phytate ratio, 1:15.

Inhibitory Potency of Phytate against Proteases

Table III-2 shows the inhibitory activities of phytate towards proteases. Phytate had inhibitory capacity against the activity of trypsin, as well as of pepsin, whereas there was a considerable difference in the capacity of enzyme inhibition among the substrate proteins examined. The substrates susceptible to the trypsin inhibition were β-lactoglobulin, gliadin, and soybean protein isolate; and the substrate susceptible to the pepsin inhibition was casein.

Table III-3 shows the effect of preincubation on the inhibition of trypsin activity by phytate. The most significant inhibition

Table III-3 Effect of preincubation on the inhibition of trypsin activity by phytate

Preincubation conditions[1]	Remaining enzyme activity (%)[2]
Without phytate	100 [a]
Preincubation [S] with [P], followed by the addition of [E]	90.6[b]
Preincubation [E] with [P], followed by the addition of [S]	77.8[d]
Preincubation [E] with [P], followed by the addition of [S] and Ca^{2+}	86.4[c]
Preincubation Ca^{2+}-stabilized [E] with [P], followed by the addition of [S]	97.6[a]

1] Both substances, under the indicated conditions of preincubation, were incubated for 10 min at 37°C and then the remaining enzyme activity was determined for 30 min at 37°C. Calcium ion was added to a final concentration of 5 mM under the indicated conditions. [E], [S], and [P] indicate the enzyme, the substrate (soybean protein isolate), and phytate, respectively. A weight ratio of these substances added was the same in Table III-1.

2] Values that do not share a common superscript are significantly different at $p<0.05$ (n=3).

among the experimental conditions tested was found when the phytate had been added to the enzyme prior to the addition of the substrate. The inhibitory activity, but less, against the enzyme was also found with the preincubation of phytate with the substrate, followed by the addition of the enzyme. On the other hand, no significant inhibition was observed by the incorporation of trypsin, which had previously been stabilized with calcium ion, into the reaction mixture of the preincubation.

DISCUSSION

Man consumes a number of plant foods containing considerable amounts of dietary tannins and phytate. Commom sources of dietary tannins and phytate include dry beans, products from cereals and legumes, and other vegetable sources. It is shown that the intake of dimetric flavans is approximately 400 mg per day in human diets from a variety of sources in most parts of the world (111). The total intake of dietary tannins may be somewhat higher than dimeric flavans. Analysis of Indian diets indicated that daily intake of tannins was in a range from 1,500 to 2,500 mg (112). Although the dietary intake of phytate is unknown, foods processed from cereals and legumes are shown to contain considerable amounts of phytate (113).

The tannins from various origins are reported to inhibit digestive enzymes (114, 115), but the detailed mechanism involved has been not clarified. The present investigation indicates that tannic acid exhibits potent inhibitory activity against trypsin (Fig. III-1) but catechin exhibits no activity. Kinetic analyses indicated that the inhibition of trypsin activity by the tannic acid conformed with a noncompetitive type. In illustration of the tannin as a protease inhibitory factor in plant food, the tannic trypsin inhibitory activity was shown to constitute approximately 16 - 22% of the total trypsin inhibitory activity in buckwheat flour

(Table III-1).

It is well known that phytate in plant foods reacts with various essential nutrients, including minerals, and makes, in many instances, them biologically unavailable for the gastrointestinal absorption (113). When bound to protein, phytate causes decreased solubility and functionality of the proteins (104-107). In view of such findings, considerable attention has been paid to removal of phytate from plant seeds on processing, particularly on preparation of protein isolate (116, 117). The present author *et al.* (118) have established the procedure using anion-exchange resin for the preparation of a protein isolate, which is of high purity and is substantially free of phytate, from rice bran (data not shown).

The present investigation showed that phytate had inhibitory capacity against the activity of trypsin, as well as of pepsin (Table III-2). The inhibitor constant, Ki, of phytate against the activity of trypsin, as assayed with BApNA as the substrate, was estimated to be 4 mM (data not shown). This value is larger than that of the tannic acid (Fig. III-1). There was, on the other hand, a considerable difference in the capacity of enzyme inhibition among the substrates examined (Table III-2). Studies of alterations of the preincubation conditions (Table III-3) suggest that phytate may interact with the substrate proteins, as well as the enzymes. The observed difference in enzyme inhibition among the substrates employed (Table III-3) may be attributed to their binding capacity towards the phytate.

CHAPTER IV

EVALUATION OF THE OVERALL INHIBITORY POTENCY OF THE ANTINUTRITIONAL SUBSTANCES TOWARDS THE PROTEIN DIGESTIBILITY OF PLANT FOODS

A growing interest in foods of plant origin, especially in plant protein food, has become evident throughout the world from the standpoint of the increasing importance as a major resource of food protein. Considerable attention has been virtually paid to the use of plant seeds as a starting material in food preparation. The nutritional quality of edible seeds is thus the subject of intense investigation (119).

The nutritional values of dietary proteins depend primarily upon the concentration and distribution pattern of their constituent amino acids. Amino acid composition data generally indicate the nutritive value of various protein sources. However, nutritive values as estimated by animal assays is often inconsistent with those as predicted from amino acid data (120). This may be largely attributed to a lack of complete biological availability for the alimentary assimilation of all of the amino acids due to incomplete digestion of the proteins. Some approaches are available for assessing the digestibility of food proteins (121, 122), but there is no consensus of opinion as the factors responsible for the protein digestibility of plant foods.

The present study was undertaken to clarify the inhibitory potency of the endogenous antinutritional factors present in plant foodstuffs towards protein digestibility and to reveal the factors responsible for the protein digestibility of various plant foods.

SECTION I

Inhibitory Potency of Plant Antinutritional Factors towards the Digestibility of Protein

The nutritional quality of protein depends both on its amino acid content and on the bioavailability of the amino acids (123). A major factor determining the bioavailability is the digestibility of protein. Thus, evaluation of the nutritional quality of food protein requires knowledge of both the amount of the constituent amino acids and of the digestibility. Information on the amino acid content of food has become reasonably adequate (124, 125), but knowledge of the digestibility of food protein is incomplete (126).

There are, on the other hand, a number of antinutritional factors adversely affecting the digestibility of the proteins in many edible seeds (Chapters I - III). These factors are likely to limit the protein digestibility through their inhibitory capacity. But, the overall inhibitory potency of these antinutrients against the protein digestibility of the plant seeds remains unanswered.

Furthermore, the question of what components in the seeds produce most significant inhibition should be resolved.

The present study was undertaken to clarify the inhibitory potency of these antinutrients towards the digestibility of protein.

MATERIALS AND METHODS

Materials

The globulin of buckwheat seeds was used as a protein sample in this study. Descriptions on the buckwheat seeds used are given in the Section III of Chapter II. The globulin was isolated from the seeds according to the procedure of Javornik and Kreft (127). The protein (N X 6.25) content of the globulin, as determined by the micro-Kjeldahl method (62), was 94.6 $\pm$ 5.0% (means $\pm$ S.D., n=4) on a dry weight basis. The globulin preparation contained 10.6 $\pm$ 0.3 mg phosphorus (means $\pm$ S.D., n=4) per 100 g solid on a dry weight basis, but no dietary fiber, as assayed by the gravimetric, enzymatic method (83), was found in the protein preparation. The endogenous, protein protease inhibitor of buckwheat seeds was prepared by the chromatographic procedure on Sephadex G-75 (See the Section III of Chapter II). Descriptions on the dietary fiber sources used are given in Chapter I. The neutral detergent fiber of buckwheat flour was prepared according to the procedure of Robertson and Van Soest (128). Statements

on the tannins and phytate used are given in Chapter III; and statements on the enzyme preparations and the substrates employed in this study are given in Cahpter I. Descriptions on the molecular-sieve gels for chromatography are given in the Sections I and III of Chapter II. All other chemicals were of analytical grade.

In Vitro Digestion

In vitro proteolytic digestion was conducted to determine the effect of the presence of selected antinutrients (protein inhibitor, dietary fiber sources, tannins, and phytate) on the concentration of free peptides released upon protein hydrolysis. The detailed conditions for the *in vitro* digestion are given in Section I of Chapter II.

An aliquot of the soluble digesta obtained on the pepsin and pancreatin digestion was applied on a Toyopearl HW-50 column (36 X 1.6 cm i.d.), which was previously equilibrated with 0.1 M Tris-HCl buffer (pH 8.0)

Assay of Enzymatic and Inhibitory Activities

The assay conditions for the enzymatic and inhibitory activities are given in Chapter I.

Determinations of Protein and Peptide

The assay conditions for protein and peptide are given in the Section I of Chapter II.

Assay of Phosphorus

Phosphorus was assayed by the method of Fiske and Subbarow (129).

Statistical Analysis

Data were subjected to analysis of variance and the significance of means was tested by Duncan's method (130).

RESULTS

Inhibition of Trypsin by Antinutritional Substances

The inhibitory capacities of dietary fiber sources, tannins, phytate, and the protein trypsin inhibitor against the hydrolytic activity of trypsin towards BApNA as the substrate are shown in Table IV-1. The protein inhibitor exhibited the highest inhibitory capacity among the substances examined. The dietary fiber sources had relatively low inhibitory capacity towards trypsin. Tannic acid exhibited a relatively high inhibitory activity towards trypsin; and phytate, a relatively low inhibitory activity. No inhibition, as assayed with BApNA as the substrate, was found with catechin.

Table IV-1 Inhibition of trypsin activity by various antinutrients

Substances tested[1]	Amounts of the inhibitors required for half inhibition (mg/mg trypsin)
Tannic acid	23.0 ± 7.2[2]
Catechin	>209
Sodium phytate	588 ± 57
Agar-agar	633 ± 10
Pectin	614 ± 88
Sodium alginate	454 ± 40
Xylan	225 ± 80
Protein inhibitor	0.89 ± 0.14

1] The enzymatic assay was performed with BApNA as the substrate.
2] Values are means ± S.D. (n=3).

Effects of the Antinutritional Substances on Protein Digestibility

The inhibitory effects of the dietary fiber sources on the peptic and pancreatic digestion of buckwheat globulin are shown in Table IV-2. The pancreatin NF used contained approximately 4.2×10^{-2} BApNA untis trypsin activity per mg solid on a dry weight basis; and approximately 2.8×10^{-2} BTpNA units chymotrypsin activity per mg solid on a dry weight basis. All the fiber sources examined, except for cellulose powder and inulin, significantly ($p<0.05$) lowered the protein digestibility. The neutral detergent fiber, prepared from buckwheat flour, was also found to reduce the digestibility of the endogenous protein. Incorporation of the protein

Table IV-2 Effects of various dietary fiber sources on the peptic and pancreatic digestion of buckwheat globulin

Dietary fiber sources	Digestibility (%)[1]	
	Weight added of fiber	
	10-fold wt to protein	20-fold wt to protein
None	34.9 ± 0.5^{ab}	34.9 ± 0.5^{a}
Agar-agar	30.2 ± 0.4^{d}	24.0 ± 0.5^{e}
Buckwheat neutral detergent fiber	31.1 ± 0.5^{cd}	31.5 ± 0.6^{b}
Cellulose powder	35.0 ± 0.1^{a}	32.9 ± 0.9^{ab}
Guar gum	30.3 ± 0.8^{cd}	28.0 ± 1.4^{cd}
Inulin	32.7 ± 0.9^{bcd}	33.9 ± 0.5^{ab}
Pectic acid	33.1 ± 0.9^{ac}	32.1 ± 0.9^{b}
Pectin	29.7 ± 0.9^{d}	25.7 ± 0.8^{de}
Sodium alginate	32.8 ± 0.4^{c}	27.7 ± 1.2^{cd}
Tragacanth gum	32.8 ± 1.3^{acd}	28.4 ± 1.0^{cd}
Xylan	31.5 ± 0.4^{cd}	28.9 ± 0.2^{c}

1] Values are means $\pm$ S.D. (n=3). Values within a column that do not share a common superscript are significantly different at $p<0.05$.

inhibitor into the digestion mixture significantly ($p<0.05$) decreased the peptic and pancreatic digestibility of buckwheat globulin (Table IV-3). Tannic acid and catechin exhibited an inhibitory effect on the protein digestibility; and phytate did not exhibit any effect (Table IV-4).

Chromatography of the digesta in the presence or absence of the antinutritional substances on a Toyopearl HW-50 column was per-

Table IV-3 Effect of the protein inhibitor on the peptic and pancreatic digestion of buckwheat globulin

Protein inhibitor added	Peptides in the digesta[1] (μg/25 mg protein)	Digestibility (%)
None	8736 ± 128[a]	34.9[a]
0.0125-fold wt to protein	8254 ± 99[b]	33.0[b]
0.05-fold wt to protein	6866 ± 148[c]	27.5[c]
0.1-fold wt to protein	4361 ± 29[d]	17.4[d]

1] Values are means ± S.D. (n=3). Values within a column that do not share a common superscript are significantly different at $p<0.05$.

Table IV-4 Effects of tannins and sodium phytate on the peptic and pancreatic digestion of buckwheat globulin

Substances added	Peptides in the digesta[1] (μg/25 mg protein)		Digestibility (%)	
	Weight added		Weight added	
	0.4-fold wt to protein	0.8-fold wt to protein	0.4-fold wt to protein	0.8-fold wt to protein
None	8736 ± 128[a]	8736 ± 128[a]	34.9[a]	34.9[a]
Catechin	8092 ± 88[b]	8107 ± 39[b]	32.4[b]	32.4[b]
Tannic acid	5738 ± 19[c]	5144 ± 74[c]	23.0[c]	20.6[c]
Sodium phytate	8661 ± 92[a]	8686 ± 180[a]	34.6[a]	34.7[a]

1] Values are means ± S.D. (n=3). Values within a column that do not share a common superscript are significantly different at $p<0.05$.

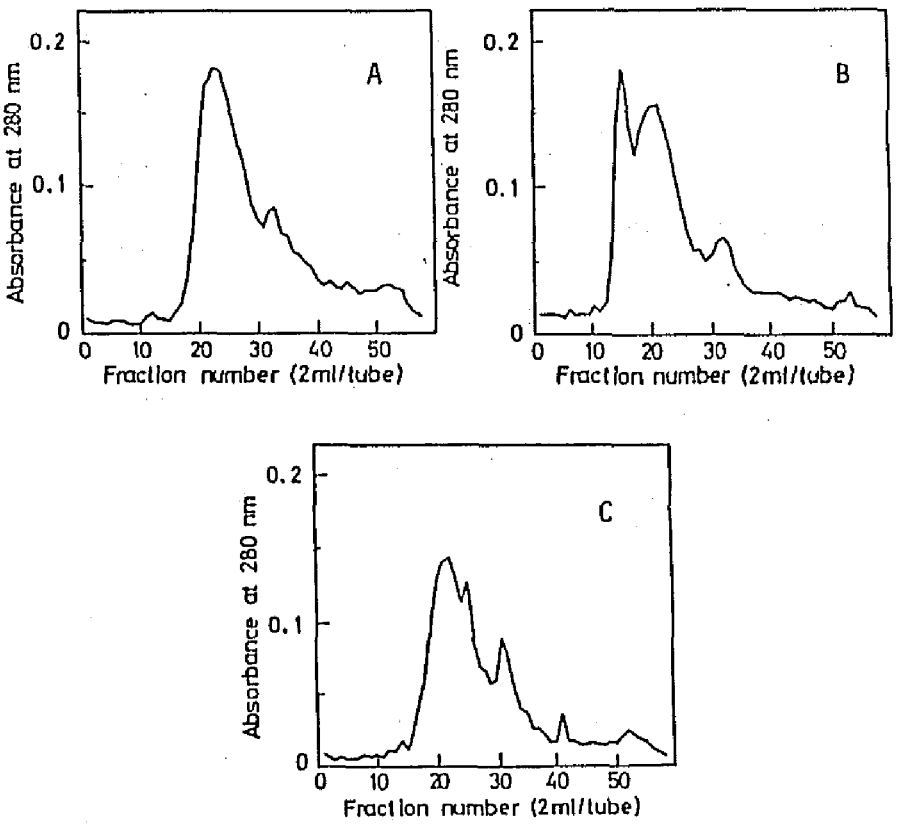

Fig. IV-1 Chromatographic elution profiles of the soluble digesta obtained on the *in vitro* digestion of buckwheat globulin in the presence or absence of antinutrients. A indicates the control digesta without antinutrients; B, the digesta with agar-agar; and C, the digesta with the protein inhibitor.

formed (Fig. IV-1). Incorporation of agar-agar into the digestion mixture led to an increase in a high-molecular-weight peptide in the digesta (Fig. IV-1B). A similar result was observed with pectin and xylan (data not shwon). On the other hand, the protein inhibitor

Table IV-5 Inhibitory potency of dietary fiber sources, tannins and protein inhibitor towards the digestion of buckwheat globulin

Substances tested	Inhibitory potency towards digestion [1] (units/mg, X 10^{-3})
Agar-agar	2.04 ± 0.21^{d}
Guar gum	1.63 ± 0.39^{ef}
Pectic acid	0.66 ± 0.27^{h}
Pectin	1.98 ± 0.29^{de}
Sodium alginate	1.16 ± 0.37^{fg}
Tragacanth gum	1.09 ± 0.41^{gh}
Xylan	1.30 ± 0.15^{fg}
Catechin	19.2 ± 7.6^{c}
Tannic acid	95.8 ± 26.4^{b}
Protein inhibitor	649 ± 63^{a}

1] One unit of inhibitor is defined as the inhibition of the conversion of 1 mg the globulin into soluble peptide under the digestion conditions employed. Values were calculated from the digestibility data of Tables IV 2-4. Values within a column that do not share a common superscript are significantly different at $p<0.05$.

did not substantially alter the chromatographic elution profile as compared with the control (Fig. IV-1C and IV-1A). Tannic acid and catechin in themselves exhibited intense absorbance at 280 nm, and, therefore these substances interfered with the direct determination of protein in column effluenct monitored 280 nm. The chromatogram, obtained from difference at 280 nm by subtraction of the enzyme-free blank, of the soluble digesta in the presence of tannic acid or catechin, indicated that these antinutritional sub-

stances also greatly altered the elution profile compared with the control (data not shown). Inhibitory potency of the antinutrients towards the digestion of buckwheat globulin was calculated from the digestibility data of Tables IV-2 to IV-4 (Table IV-5). The protein inhibitor exhibited the highest inhibitory potency among the substances examined.

DISCUSSION

The nutritive value of dietary proteins depends on the biological availability of their amino acids. Although the presence of antinutrients, as well as the relative proportions of the constituent amino acids, profoundly affects the nutritive value of edible seeds, there were few attempts to determine the correlation between the level of antinutrients in edible seeds and their overall biological impact (131, 132).

The inhibitory potency of dietary fiber sources, tannins, phytate, and the protein trypsin inhibitor towards the pepsin-pancreatin digestibility of protein was compared (Tables IV-2 - IV-4). The protein inhibitor exhibited the highest inhibitory capacity among the substances examined (Table IV-5). Both tannins exhibited a pronounced inhibitory effect on the digestibility of protein, whereas no inhibition was found with phytate.

Legumes and cereals, on the other hand, contain 45 - 100 mg pro-

tein protease inhibitors per 100 g seeds, representing 2.5% or more of the whole seed protein (2). They also contain 0-1.6% tannins (133), and the pigmented varieties contain 2-4% condensed tannins (134). In addition, the plant seed contain 9.3-23.2% dietary fiber as measured by the gravimetric, enzymatic method (83, 135, 136). In view of the observed inhibitory potency of these antinutrients against the digestion (Tables IV-2 to IV-5) and of their levels in edible seeds, the inhibition of the digestion of dietary sources of plant proteins by such factors as dietary fiber and tannins, in addition to the protein protease inhibitors, should also be taken into consideration.

SECTION II

Evaluation of the Overall Inhibitory Potency of the Antinutritional Substances towards the Protein Digestibility of Plant Foods

Exact evaluation of the nutritional quality of food protein is a major problem in human nutrition. Since biological assays are an expensive and time-consuming procedure, many researchers have tried to develop *in vitro* methods of assessing the protein quality for many years. Although many correlations have been made between amino acid composition and biological data (126), there is still no clear understanding at overall correlations. Current evidence suggests that the most appropriate approach for evaluating the quality of protein is one based on the amino acids adjusted for the bioavailability of the protein (137). Protein digestibility is a major factor determining the bioavailability of the protein, and thus factors responsible for the protein digestibility of food should be resolved, probably proving to be of considerable importance of the discrepancies between amino acid scores and biological data.

The present study was undertaken to clarify the inhibitory factors responsible for the protein digestibility of various plant foods.

MATERIALS AND METHODS

Materials

Nine different kinds of plant foods were selected for this in-

vestigation: barley flour, buckwheat flour, ground tea (maccha), polished rice, soybean meal, roasted and ground soybean (kinako), soybean protein-lipid film (yuba), rice bran, and wheat flour. These foods were obtained locally and stored at -35°C before use. Polished rice was cooked under recipe conditions before analysis.

Assay of Trypsin Inhibitory Activity

The assay conditions for trypsin inhibitory activity are given in Chapter I.

Determinations of Dietary Fiber, Tannin, and Protein

The assay conditions for protein are given in Chapter I; for tannin, Chapter III; and for dietary fiber, the Section III of Chapter II.

In Vitro Digestion

The assay conditions for *in vitro* digestion are given in the Section I of Chapter II.

Statistical Analysis

Descriptions on statistical analysis are given in the Section I of this chapter.

Table IV-6 *In vitro* protein digestibility of various plant foods

Food tested	Protein digestibility (%)[1]
Barley flour	39.6 ± 1.8^{c}
Buckwheat flour	49.7 ± 2.6^{b}
MACCHA, Ground tea	32.4 ± 2.8^{d}
Polished rice, cooked	49.3 ± 4.3^{b}
Raw soybean meal	30.9 ± 1.2^{d}
KINAKO, Roasted & ground soybean meal	48.9 ± 3.3^{b}
YUBA, Soybean protein-lipid film	67.3 ± 3.0^{a}
Rice bran	36.0 ± 4.2^{c}
Wheat flour	63.3 ± 7.8^{a}
Casein	69.2 ± 6.2^{a}

1] Values are means $\pm$ S.D. (n=6). Values that do not share a common superscript are significantly different at $p<0.05$.

RESULTS AND DISCUSSION

Table IV-6 shows the digestibility of the proteins in various plant foods. The foods examined exhibited different levels of digestibility, respectively. Wheat flour exhibited a similar, high level of protein digestibility as with casein. On the other hand, raw soybean meal and rice bran exhibited considerably low protein digestibility. Ground tea (maccha), which was rich in tannin, also had low digestibility.

Table IV-7 shows the trypsin inhibitory activities in the plant foods whose protein digestibility had been examined (Table IV-6)

Table IV-7 Trypsin inhibitory activity in various plant foods

Food tested	Trypsin inhibitory activity (IU per 3g flour)
Barley flour	7.7 ± 0.4[1]
Buckwheat flour	24.1 ± 1.5
MACCHA, Ground tea	3.0 ± 0.2
Polished rice, cooked	Nil
Raw soybean meal	255.1 ± 8.0
KINAKO, Roasted & ground soybean meal	0.2 ± 0.1
YUBA, Soybean protein-lipid film	7.8 ± 0.6
Rice bran	9.2 ± 0.2
Wheat flour	1.5 ± 0.1

1] Values are means ± S.D. (n=3).

Raw soybean meal exhibited the highest trypsin inhibitory activity among the food examined. Rice bran and barley flour, which exhibited low protein digestibility (Table IV-6), had a rather low level of trypsin inhibitory activity (Table IV-7). As earlier suggested in the Section II of Chapter II, the protein digestibility of plant foods may not conform with only a function of the trypsin inhibitory activity present.

The contents of dietary fiber, tannin, and 0.2 M sodium chloride-soluble protein in the plant foods examined here have been assayed.

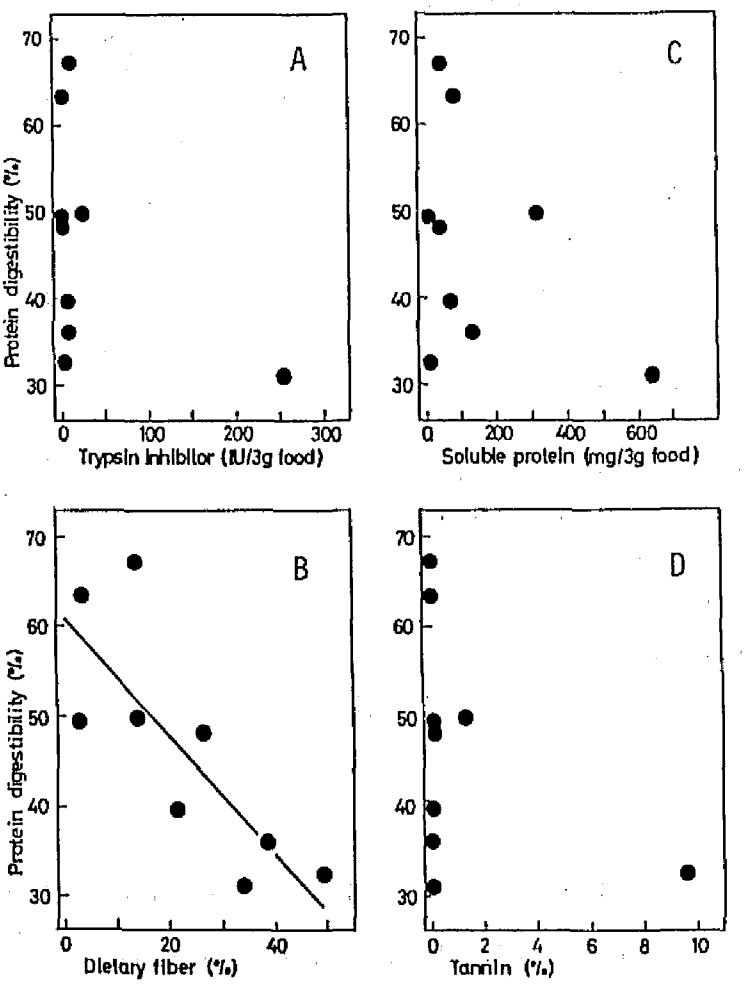

Fig. IV-2 Diagram relating trypsin inhibitor activity, dietary fiber content, soluble protein content, and tannin content to the protein digestibility of various plant foods. A indicates the diagram with trypsin inhibitor activity; B, with dietary fiber content; C, with soluble protein content; and D, with tannin content.

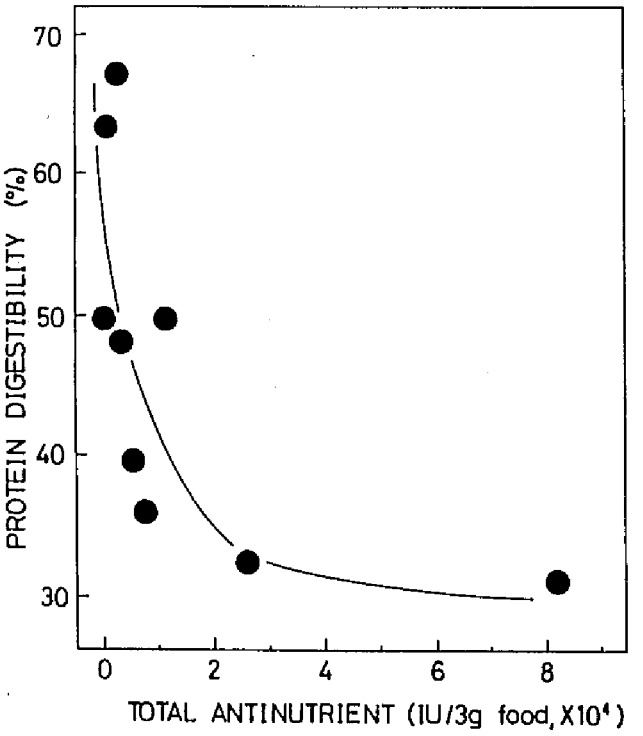

Fig. IV-3 Relationship of the overall activity of the endogenous antinutritional factors in various plant foods towards their protein digestibility

Figure IV-2 illustrates the scatter diagrams relating the trypsin inhibitory activity (Table IV-7), and the assayed contents of dietary fiber, tannin, and soluble protein to the protein digestibility of the plant foods (Table IV-6). Any relationship of the trypsin inhibitory activity, the tannin content, and the soluble protein content towards the protein digestibility was not found. There was, on the contrary, a relatively high correlation between the digestibility and dietary fiber (correlation coefficient γ = - 0.811).

On the other hand, the relative inhibitory potency of dietary fiber sources, tannins, and protein inhibitor towards protein digestibility has been revealed in the Section I of this chapter: dietary fiber was estimated to exhibit approximately 1.2 inhibitor units; tannins, approximately 20 inhibitor units; and protein inhibitor, approximately 600 inhibitor units. Now attempts have been performed to evaluate the overall inhibitory potency of these antinutrients in the plant foods against protein digestion from their respective inhibitor potency and from the assayed levels in the foods.

Surprisingly enough, there was a clear relationship of the overall inhibitory activity of the endogenous antinutrients in the plant foods towards their protein digestibility (Fig. IV-3). This finding strongly suggests that the digestibility of the proteins in plant foods conforms to a function of the overall inhibitory potency of their inherent antinutrients. This also suggests that the overall inhibitory potency of the antinutrients in plant foods should be taken into consideration on the evaluation of their protein quality, so proving to be of considerable importance of the discrepancies between amino acid scores and biological data.

There are a number of unanswered questions concerning the nutrition of food protein, and the present data may hopefully stimulate further investigation to fully answer them.

SUMMARY

The present investigation focused on the systematic understanding of the inhibitory factors involved in the protein digestibility of plant foodstuffs.

Chapter I:

The present study was undertaken to identify dietary fiber as an inhibitory factor against protein digestibility. Many of dietary fiber sources exhibited significant inhibitory activity towards proteolytic enzymes. Kinetic analyses showed that the inhibition of trypsin activity by the fiber sources conformed to a parabolic non-competitive type. The data obtained suggest that the dietary fiber sources, as they combine with the substrate protein, may render the protein poorly available for proteolytic action. The nutritional significance of the observed enzyme inhibition by dietary fiber sources was discussed.

Chapter II:

The present study aimed to reveal the effects of food processing on the protein digestibility and protease inhibitor in edible seeds. Two different samples consisting of plant seeds as major sources of dietary protein were selected for this study: soybean and buckwheat.

Two experimental facts were confirmingly demonstrated indicating that the trypsin inhibitory activity decreased during germination of soybean seed; and that the protein digestibility of the seedling was significantly higher than that of the dormant seed. Chemical analyses indicate that an improvement in the protein digestibility may be due to alterations of the protein components of soybean during germination rather simply to the decreased antitryptic activity.

Another study was performed to reveal the factors involved in the protein digestibility of several traditional soybean foods. The protein digestibility of the soybean foods did not conform to only a function of the inherent protease inhibitory activity. It is here suggested that, in addition to the protease inhibitor, other factors involved in the protein digestibility should be taken into consideration.

Nutritional properties of buckwheat were presented in this study. An aminopeptidase was identified and characterized as a possible factor affecting the product-making quality of the seed. The present study showed the occurrence of protein protease inhibitors in buckwheat. Possible nutritional significance of the inhibitors was discussed.

Chapter III:

The present study was undertaken to reveal the inhibitory potency

of tannins and phytate against protein digestibility. Tannic acid was shown to exhibit potent inhibitory activity against trypsin, whereas phytate exhibited relatively low inhibitory activity. Possible roles of both substances as an antinutrient was discussed.

Chapter IV:

The present study aimed to clarify the inhibitory potency of plant antinutritional factors towards the digestibility of protein. The inhibitory potency of dietary fiber sources, protein inhibitor, tannins, and phytate towards the pepsin-pancreatin digestibility of protein was compared. The protein inhibitor exhibited the highest inhibitory capacity among the substances tested; and phytate the lowest. The present study suggests that the inhibition of the digestion of dietary sources of plant proteins by such factors, as dietary fiber and tannins, in addition to the protein inhibitors, may also be taken into account.

Studies were conducted to clarify the relationship of plant antinutrients with protein digestibility. Demonstrated is in this study that there is a clear relationship of the overall inhibitory activity of the endogenous antinutrients in the plant food towards their protein digestibility. This finding indicates that the digestibility of the proteins in plant foods conforms to a function of the overall inhibitory potency of their inherent antinutrients.

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